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PURIFICATION OF DIPHTHERIA TOXOID.

C. G. POPE AND F. V. LINGGOOD.

From the Wellcome Physiological Research Laboratories, Langley Court, Beckenham, Kent.

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THE problem of producing diphtheria toxoid in a state of purity is of importance both for work on its chemical constitution, and because toxoid is used on a large scale for human immunization against diphtheria. During the last few years the work of Eaton (1936) and of Pappenheimer (1937) has resulted in the purification of diphtheria toxin, and its isolation in what would appear to be a pure condition. It has the properties of a protein, easily denatured by acid and heat-coagulable, with an isoelectric value of about pH 4.

Through the action of formaldehyde, under controlled conditions, toxin may be converted into a non-toxic derivative, with little loss of antigenic value or speed of flocculating with antitoxin. The changes in the chemical constitution of the toxin as a result of the action of formaldehyde are not known, but with various types of medium (tryptic digest, semi-synthetic, peptone or Martin's medium), some change, either in the toxin itself or in some constituent of the medium, renders inapplicable the methods of purification used by Eaton and Pappenheimer. We have had no experience, however, with toxoid prepared from Pappenheimer's synthetic medium.

As a result of Pappenheimer's work we now have available certain data which may be applied to a study of suitable methods for the purification of toxoid. He found that the isolated toxic protein contained 0·00046 mg. of nitrogen per Lf unit, and Pappenheimer and Robinson (1937) showed that a similar value could be deduced from the nitrogen content of floccules produced as the result of interaction between diphtheria toxin and its antitoxin. If it is assumed that the conversion of toxin into toxoid by formaldehyde does not involve any change in the nitrogen content, then this value of 0·00046 mg. N/Lf may be taken as a basis for purity determinations for toxoid. Since the toxic protein has been shown to be heat-coagulable we have employed the following method: Knowing the number of Lf units contained in a given volume of toxoid or toxin, it is possible to calculate from the above value the amount of heat-coagulable nitrogen which should be found if the only heat-coagulable protein present were specific toxin or toxoid. Generally there is also present

heat-coagulable protein derived from the bacillus due to autolytic changes. However, the ratio $\frac{\text{Theoretical toxin nitrogen}}{\text{Found coagulable nitrogen}}$ gives an indication of the purity of the material in terms of protein content. The nitrogenous materials, other than proteins, which may be present give rise to far less difficulty in the purification processes; they can be estimated by the difference between the total nitrogen and the coagulable nitrogen figures.

Several methods for the partial purification of toxoid have been reported, but only one worker, Boivin (1937), has claimed a product of the same order of purity as that described by Eaton and Pappenheimer for toxin. We have attempted the method described by Boivin (trichloracetic acid precipitation at pH 4) without success, and moreover, in view of Eaton's findings regarding the ease with which toxin is denatured in slightly acid solutions, such a method would be undesirable for our purpose. The observations of our colleague, Mr. Glenny, support those of Eaton, for he has found almost invariably that toxoids partially purified by methods which have involved an acid treatment are poor antigens when used on horses. The method used by Schmidt and Hansen (1933) is far less drastic, since it only involves the adsorption of non-specific nitrogen from the toxoid by means of charcoal, the adsorption of the toxoid and other protein with Willstätter aluminium hydroxide and its elution from the hydroxide with phosphate solution. This method, when applied to our toxoids, has never given us a product which contained more than about 500 Lf units per mg. of nitrogen.

The work reported in this paper deals with attempts which we have made to purify toxoid by methods which do not involve any drastic treatment which would be liable to cause denaturation and loss of antigenic function.

EXPERIMENTAL.

Conditions for Heat-Coagulation of the Proteins.

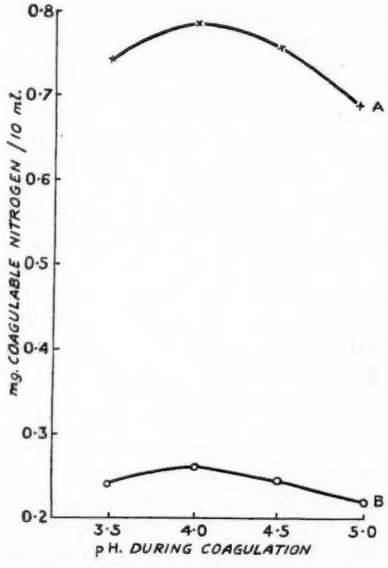
The method for determination of the total heat-coagulable proteins present in either toxins or toxoids as finally worked out was as follows: To the material was added 2 per cent. sodium chloride and 0·5 per cent. sodium acetate; the pH value was then adjusted to 4·0 with acetic acid (using a glass electrode). Duplicate or triplicate amounts of 10 or 20 ml. (depending on the amount of protein present) were heated in centrifuge tubes to 100° C. for at least five minutes. The resulting precipitates were centrifuged down, washed repeatedly with distilled water, and the nitrogen content of the precipitate determined by a micro-Kjeldahl technique.

Curves I and II show that for toxins and toxoids the optimal value for the determination of the coagulable proteins is at pH 4·0.

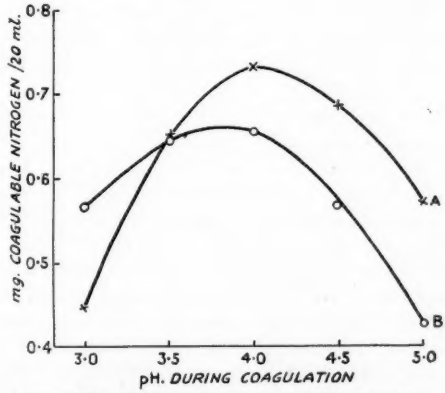
The Coagulable Protein Ratio of Toxins and Toxoids.

The method of investigation described was applied to a number of toxins and toxoids produced on the usual types of medium. Table I shows the results which were obtained. The strains of *C. diphtheriæ* used were all P.W.8,

obtained at various times from different laboratories, and all the media were prepared by tryptic digestion of meat, except for that produced with Difco peptone and the Martin's medium. It will be seen from the table that the figure for the ratio $\frac{\text{Theoretical toxin nitrogen} \times 100}{\text{Coagulable nitrogen found}}$ varied in this series from about 20 to 60. No correlation could be found between the type of medium, strain or conditions of growth and the ratio figure determined in this way. Generally the ratio figure lies between 20 and 35, and it is an exception to find values higher than these.



CURVE I.—Showing the optimal pH for coagulation of protein in toxins. A. Crude toxin. B. Partially purified toxin.



CURVE II.—Showing the optimal pH for coagulation of protein in toxoids. A. Crude toxoid. B. Partially purified toxoid.

The presence of coagulable protein, other than toxin protein, in these culture filtrates makes more difficult the problem of purification. We were somewhat surprised at the large amount of protein contamination which was revealed by this method. The greater part of the non-specific protein appears to arise from bacterial autolysis during growth, yet culture filtrates examined at varying growth periods up to 10 days did not show any substantially different ratio figures.

Methods of Purification.

As possible methods for the purification of toxoids we have tried ultrafiltration, treatment with charcoal and adsorption with Willstätter aluminium hydroxide, either singly or in combination.

Ultrafiltration has been used for this purpose by Wadsworth and Quigley

TABLE I.—*Showing the Percentage Ratio* $\frac{\textit{Theoretical Toxin N}}{\textit{Found Coagulable N}} \times 100$ *found in* C. diphtheriæ *Culture Filtrates Grown on the Usual Type of Broth Medium with high nitrogen contents*).

Material.	Type of broth.	P.W. 8 sub-strain.	Lf/ml.	Coag. N. mg./10 ml.	Per cent. ratio.
Toxin	Tryptic digest of bullocks' hearts	110	20·5	0·535*	20·1
,,	ditto	L3	53·5	0·770	34·8
,,	,,	1058	33·5	0·675	24·8
Toxoid	,,	L4	29·5	0·304	48·6
Toxin	Tryptic digest of beef	1058	61	0·862	35·5
Toxoid	ditto	1058	57	0·475	60·0
,,	,,	L3	55	0·907	30·4
Toxin	,,	L3	61	0·785	38·9
Toxoid	Tryptic digest of horse muscle	1058	49	0·749	32·8
,,	ditto	1058	57	0·831	34·6
,,	Veal infusion Difco proteose	1058	42·5	0·621	34·3
,,	Martin's	L3	24·5	0·514	34·9
,,	,,	1058	36·5	0·799	24·1
,,	,,	1058	35·5	0·383	46·3

* Mean values.

(1934), and we have found it to be a useful method for the removal of the smaller nitrogenous materials. With the usual type of ultrafilter, consisting of an alundum candle impregnated with acetic collodion, we have found that if the filter retains the toxoid, it also retains the non-specific proteins, and no increase in ratio results from ultrafiltration. For this work we have tried a number of forms of collodion, including special types of low viscosity, but have reached the conclusion that the material used by Wadsworth and Quigley known as parlodion is as satisfactory as any. Generally an 8 per cent. solution of this brand of collodion in acetic acid gives a membrane which retains the toxoid. On some occasions toxoids have been encountered which ultrafilter with difficulty owing to the formation on the filter of a gummy layer. This difficulty has generally been found with toxoids prepared from veal media or Witte peptone. When the greater part of the liquid has been removed by ultrafiltration, washing may be carried out with 0·25 per cent. phenol-saline, but owing to the denaturing effect of phenol it is preferable to use for washing a phenol-saline containing 1·0 per cent. sodium bicarbonate.

Schmidt and Hansen (1930) have used charcoal for the partial purification of toxoid prior to adsorption on aluminium hydroxide, and we have examined the action of charcoal of various makes on crude toxoid. One of the most satisfactory makes used by us is " Eponit ". Crude toxoids vary enormously in their properties as judged by the action of various amounts of charcoal at constant pH values. In some toxoids the addition of a suitable amount of

charcoal removes a considerable amount of colour and nitrogen, and increases the ratio with only a small loss of specific toxoid. The action of charcoal is not sufficiently consistent to make it of much value for purification used alone, although combined with ultrafiltration it has proved useful. After charcoal treatment ultrafiltration proceeds much more rapidly.

By a combination of these methods some degree of increase in purity was obtained. In practice, treatment with charcoal was first applied to the crude toxoid, after which it was ultrafiltered and the concentrated material washed on the ultrafilter with bicarbonate-phenol-saline. Material partially purified in this way shows a marked difference in its behaviour towards Willstätter aluminium hydroxide compared with crude toxoid. For the adsorption of nearly 100 per cent. of the specific toxoid present in the crude material it is necessary to add about one-fifth volume of hydroxide (containing 1·5–2·0 per cent. $Al_2(OH)_3$), whereas partially purified materials containing about 100 Lf units per ml. are adsorbed by approximately 25 ml. of aluminium hydroxide per litre of toxoid. By careful elution with 2 per cent. disodium phosphate solution the toxoid may be recovered from the aluminium hydroxide in purer form. These methods failed to give a purified toxoid having nitrogen/Lf value similar to that reported by Pappenheimer for toxin, and we therefore turned our attention to the problem of producing a crude toxoid which would be more suitable for subsequent purification.

Preparation of Toxoid Suitable for Purification.

The methods employed in this work had shown that the crude toxoids contained a considerable amount of non-specific heat-coagulable protein, and that the removal of this material presented the greatest difficulties in methods used for purification. We therefore aimed at the production of culture filtrates of *C. diphtheriæ* in which this non-specific protein was minimal in amount. After many unsuccessful attempts we made an observation on toxin production in medium of low nitrogen content which proved to be very important. For many years the nitrogenous content of culture media was considered to be very important for toxin production, and it is only comparatively recently that the value of certain carbon compounds for the production of high value toxins has been known. In media containing a high nitrogen content (50–60 mg. of total nitrogen per 10 ml.) and energy sources (sodium acetate, lactate and maltose), *C. diphtheriæ* grows very rapidly at the surface of the medium. There is also produced a large growth of organisms which settle on the bottom of the culture flask or bottle. It has been shown (Pope and Healey, 1933) that toxin is only produced in measurable quantity by those organisms which are growing at the surface, and it was thought that these non-toxin producing deposits lying at the bottom of the medium were the probable source of much of the non-specific protein which arose from autolysis of the organisms. Experiments with media* containing a variable nitrogen content and a constant energy source content showed that it was possible to obtain conditions such that the organism grew in pellicle form with little or no deposit. An examination of the

* See Appendix.

culture filtrates produced in this way showed that the proportion of non-specific protein to specific protein had been greatly reduced. Table II shows the results obtained with culture media of such low nitrogen content that the organism grows in pellicle form without deposits. Two sub-strains of *C. diphtheriæ* P.W.8 were used, and it will be seen that the toxin production was quite good for a special medium of this type. Minor modifications made to the medium later in the work resulted in stronger toxin production without sacrifice of the desirable properties of the medium. The ratio figures (duplicate results) show a considerable improvement over those shown in Table I.

TABLE II.—*Showing the Percentage Ratio for* C. diphtheriæ *Culture Filtrates Grown on Special Low Nitrogen Medium.*

P.W. 8 sub-strain.	Lf./ml.	Coag. N. mg./10 ml.*	Per cent. ratio.
L3	24·5	0·217	56·6
,,	24·5	0·216	56·8
,,	26·5	0·297	46·5
,,	22·5	0·215	52·4
,,	35·5	0·364	48·8
,,	26·5	0·257	51·7
,,	22·5	0·160	70·4
,,	23·5	0·206	57·1
1058	33·5	0·411	40·9
,,	38·5	0·366	52·7
,,	47	0·530	44·5
,,	43	0·606	35·6
,,	37·5	0·275	68·4
,,	39·5	0·436	44·6

* Mean values.

The advantages of this low nitrogen medium, which gives a higher ratio figure, are not restricted to the crude toxoid stage, for it was found that charcoal treatment of the toxoids led to great increases in purity. Table III shows some results obtained from charcoal treatment of toxoids of this type. Although a part of the specific toxoid is lost, the purity in terms of coagulable protein may be increased to nearly 100 per cent. Such material still contains nitrogen due to the presence of non-protein nitrogenous compounds, the greater part of which may be removed by ultrafiltration followed by washing. The table shows that after such treatment toxoids containing about 1200 Lf units per mg. of total nitrogen can be prepared. Most of the contaminating nitrogen present is in the form of proteose-nitrogen.

After the special toxoids have been partially purified in this way a little additional purification may be obtained either by precipitation with potash alum or by adsorption with Willstätter aluminium hydroxide. Elution of the toxoid with 2 per cent. disodium phosphate has given products containing up to 1500 Lf units per mg. of nitrogen. We have found it better to make only one elution with phosphate, for although some additional toxoid can be eluted by a second washing, the material is always less pure.

TABLE III.—*Showing the Effect of Charcoal and Ultrafiltration in Increasing the Purity of Toxoids Prepared with the Low Nitrogen Medium.*

Lf/ml.	Per cent. ratio.	Per cent. charcoal.	Lf/ml.	Per cent. ratio.	Purity on ultrafiltration. Lf units/mg. N
21·5	44	2	20·5	..	
		3	19·5	..	
		4	18·5	75	
		5	15·5	95	1210
23·5	55	3	18·5	91	
		4	13·5	104	
		5	4	..	
		3·5	..	..	1200
36·5	50	3	33·5	62	
		4	31·5	70	
		5	28·5	78	
		6	23·5	95	
		7	14·5	100	
22·5	54	3·5	16·5	90	
45·5	44	4	37·5	64	
		5	34·5	73	
		6	28·5	84	1150

The purity of the material which we have obtained (approximately 1500 Lf units per mg. nitrogen) is less than that obtained by Pappenheimer for toxin (2160 Lf units per mg. nitrogen), and for this reason we have not carried out extensive work on its chemical properties. Nevertheless, the methods employed are free from certain objections which may be raised against other procedures, and should cause no loss of antigenic properties. For this reason the purified material should be preferable for use in the production of alum precipitated toxoid for human immunization. Work on this point and on methods which will give a pure product is in progress.

APPENDIX.

Preparation of Culture Medium.

The method used is based on that described by Pope and Smith (1932), with the modifications detailed below.

Beef muscle, freed from excess fat and minced, is added at the rate of 33 lb. to 50 litres of tap-water and heated with constant stirring to 80° C. A further 100 litres of cold tap-water are then added, the temperature adjusted to 50° C. and the pH to 8·0, using powdered sodium carbonate. The tryptic digestion is carried out, using an acid-alcohol emulsion of minced ox pancreas prepared by the method of Cole and Onslow (1916). For the first hour 400 ml. of emulsion are added half-hourly, and for the remaining 3·5 hours 200 ml. quantities are added half-hourly. The pH is maintained throughout at 8·0 using 10 *N* NaOH. After 4·5 hours' digestion, 1500 ml. (1·0 per cent.) glacial acetic acid are added and the digest boiled vigorously for 30 minutes, any water lost during boiling being replaced. The material is then filtered on filter cloth bags. After warming the filtrate to 30° C. the pH is adjusted to 8·0 and 500 g. of yeast

added (the yeast is washed on the centrifuge before use). After 2 hours' fermentation at 30° C., the temperature is raised to 70° C., 0·2 per cent. of 50 per cent. sodium lactate and 0·3 per cent. maltose added and the pH carefully adjusted to 8·0. The medium is then paper-filtered. Sterilization is effected by filtration through Berkefeld candles followed by heating to 100° C.

Medium prepared in this manner contains about 0·20 to 0·24 per cent. of total nitrogen, of which about 30 per cent. is amino-nitrogen.

The crude trypsin preparation sometimes varies in activity and may produce medium with higher nitrogen figures. In such medium, growth and toxin production is increased, but owing to the production of "deposited bacteria" during growth the ratio figure falls to a low value. For such active trypsin preparations the amounts of emulsion used should be reduced until the nitrogen figures agree with those quoted above.

SUMMARY.

(1) Based on Pappenheimer's value of 0·00046 mg. nitrogen per Lf unit for toxin and the fact that the toxin appears to be heat-coagulable protein, we have measured the heat-coagulable protein present in diphtheria toxins and toxoids, and expressed the toxin protein as a percentage of the total protein found.

(2) The optimal value for heat-coagulation in both toxins and toxoids was found to be pH 4.

(3) The method showed that in culture filtrates as usually prepared, the specific toxin protein accounted for about 20–35 per cent. of the total protein found. These crude toxoids responded poorly to the methods employed for purification.

(4) The preparation of culture filtrates on a medium of low nitrogen content is described. In these filtrates the specific toxin accounts for 50 per cent. or more of the total protein.

(5) By treatment with charcoal, the ratio can be increased so that the specific toxoid accounts for nearly 100 per cent. of the coagulable protein.

(6) Further purification was obtained by ultrafiltration, washing the product on an ultrafilter with bicarbonate-phenol-saline solution, adsorption on aluminium hydroxide and elution with disodium phosphate. Toxoids containing nearly 1500 Lf units per mg. nitrogen have been obtained in this way.

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THE EFFECT OF FOREIGN TISSUE EXTRACTS ON THE EFFICACY OF INFLUENZA VIRUS VACCINES.

C. H. ANDREWES AND WILSON SMITH.

From the National Institute for Medical Research, Hampstead, London, N.W. 3.

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A NUMBER of workers have recorded that virus vaccines made from the tissues of infected animals are effective immunizing agents only so long as they are used within the same animal species. Thus Laidlaw and Dunkin (1928) found that formolized dog-distemper vaccines made from dog-tissues were of relatively little value for immunizing ferrets and *vice versa*. Traub (1938) also reported that formolized mouse-tissue vaccine was far inferior to guinea-pig-tissue vaccine for immunizing guinea-pigs against the virus of lymphocytic chorio-meningitis. Throughout our work on human influenza virus we have always failed to immunize mice with vaccines made from ferret-tissues. Shope (1936) has studied the question as regards swine influenza virus : in his experience the virus-containing tissues of ferrets and swine have failed to immunize mice, when injected subcutaneously, though effective in the homologous species ; similarly, swine and mouse vaccines have failed in ferrets, though, curiously enough, vaccines made from any of the species concerned would protect swine.

In attempting to immunize animals against influenza virus it has become evident that the outcome is determined by a number of factors, the relative importance of which is often difficult to assess. Amongst these are (i) the titre of the virus used to make the vaccine ; (ii) the animal source of the vaccine ; (iii) the route of inoculation ; and (iv) the state of the virus, whether living, heated or inactivated with formaldehyde.

METHODS.

For the immunization of mice we injected young animals, usually in groups of 12, with 0·2 c.c. of an influenza virus vaccine (W.S. strain) ; the route of inoculation varied from one experiment to another. Fourteen days later, 0·5 c.c. of similar material was given, and after a further fortnight the mice were tested for immunity by inoculating diluted virus filtrate intranasally under ether anæsthesia. All mice dying were examined *post mortem* ; 14 days after the immunity test survivors were killed and similarly examined. The virus preparations used for immunization were titrated intranasally in mice ; they consisted either of filtrates of infected mouse-lungs, centrifuged 5 per cent. suspensions of infected ferret-lungs, or centrifuged 48-hour tissue cultures of influenza virus in minced chick embryo-Tyrode medium. Virus used for immunity tests always consisted of mouse-lung filtrates and was also titrated in mice.

For immunizing ferrets we used centrifuged mouse or ferret lung suspensions, and gave 2 doses of 5 c.c. subcutaneously a fortnight apart ; intranasal immunity tests with centrifuged ferret virus were made 14 days after the second inoculation. As recorded in previous papers (Smith, Andrewes and Laidlaw, 1935), no vaccinating procedure could be relied upon to produce complete immunity in the ferret ; the effect of immunization could best be judged by noting whether or not lung lesions were prevented. Variations from these routine methods will be recorded in the text or tables.

ANALYSIS OF FACTORS AFFECTING THE RESULTS OF IMMUNIZATION.

(i) *Virus Titre.*

In comparing the immunizing value of different vaccines in the ferret and mouse one must, naturally, take into account the titre of the virus used to make the vaccine. It is not easy to decide how to measure this, for virus repeatedly passed through ferrets commonly has a higher titre for ferret than for mouse, while the contrary is true for mouse-adapted virus. Thus one ferret-virus filtrate was effective at a dilution of $1 : 10^6$ when tested in ferrets but only at $1 : 10^4$ in mice. One filtrate of mouse-adapted influenza virus had a titre of $>1 : 10^2 <1 : 10^4$ in ferrets but $1 : 10^5$ in mice ; another a titre of $1 : 10^2$ in ferrets and $1 : 10^6$ in mice. It is thus not necessarily correct to regard as comparable (as Shope does (1936)) virus preparations having a similar titre for mice, irrespective of whether they are derived from mouse, ferret or pig. We have thought it fairer to record in our tables the titre for the more susceptible (or homologous) species. An added complication is introduced in attempting to titrate mouse virus in ferrets, by the fact that highly diluted mouse virus given intranasally may immunize ferrets without provoking any fever or symptoms, thus behaving like Burnet's (1937) egg-adapted virus. Dilutions of ferret-adapted virus, on the other hand, have either produced in ferrets both symptoms and subsequent immunity or have produced neither. The behaviour of the dilute mouse virus in ferrets has, unfortunately, been so irregular as to make it difficult to study.

Virus cultivated on chick-embryo tissue-cultures has been titrated on mice ; we have not practised the method of pock-counting on chorio-allantoic membranes as described by Burnet. We have, however, attempted to compare virus titres by estimating the antibody-absorbing power of virus preparations (*cf.* Smith and Andrewes, 1938) ; such a method might be expected to be independent of any complications caused by the differing susceptibilities of various hosts. The results did not indicate that the amount of virus in our tissue-culture virus was any more than the results of the mouse titration had suggested. Both with tissue-cultures and with mouse-virus suspensions the highest dilution which could be shown to absorb antibody was 10,000 (rarely 100,000) times as concentrated as the minimal infective dilution for mice. For instance, a virus tissue-culture having a titre, for mice, of $1 : 10^5$, absorbed antibody from a dilute immune ferret serum only when diluted 1 : 10 or less. This virus was of the mouse-adapted W.S. strain only propagated for a short time in chick tissue-cultures.

TABLE I.—*Ferret vs. Mouse Vaccines for Immunizing Mice.*

Source of virus.	Titre of virus in the two vaccinating doses.	Route of inoculation.	Result of immunity test.			
			No. of mice in group.	No. dying of influenza.	No. of survivors.	
					With lung lesions.	Without lung lesions.
Ferret lung (unfilt.)	10^2 and 10^4	s.c.	12	12	0	0
,, ,,	,,	i.p.	11	8	0	3
,, 1 in 10	10 and 10^3	i.p.	10	9	1	0
Mouse lung filtrate	10^6 and 10^6	s.c.	11	7	1	3
,, ,, 1 in 10	10^5 and 10^5	,,	11	10	0	1
,, ,, 1 in 100	10^4 and 10^4	,,	11	11	0	0
,, ,, 1 in 10	10^5 and 10^5	i.p.	11	4	5	2
,, ,, 1 in 100	10^4 and 10^4	,,	9	8	0	1
Nil (controls)	..	..	10	10	0	0

s.c. = Subcutaneous.
i.p. = Intraperitoneal.

(ii) *Animal Source of the Vaccine.*

Mouse vs. ferret virus as vaccine for mice.

Table I shows that unfiltered living ferret virus is of practically no value for immunizing mice, while living mouse-virus filtrate even when diluted 1 : 10 produces an effect, at any rate when given by the intraperitoneal route. The evidence in Table I concerning the importance of the route of inoculation will be considered later.

Mouse vs. ferret virus as vaccine for ferrets.

In contrast to the mice, ferrets have, in our experience, been equally well immunized by living mouse or ferret vaccines. Experiments showing this have been previously published (Stuart-Harris, Andrewes and Smith, 1938). Another result of the same kind is shown in Table II.

As Table I shows, the titres of the mouse viruses were higher than those of the ferret viruses, even taking the " titre in the more susceptible species " as guide. One could thus explain on the basis of titre alone that ferret virus should be relatively poor in the mouse, and that mouse virus should be effective even in the ferret. It is unlikely, however, that everything can be thus explained, for the ferret material, despite its low virus content, does immunize the ferret when it cannot immunize the mouse ; and the mouse virus, despite its much higher titre is, in the ferret, scarcely more effective than the ferret virus (*cf.* Table II). Shope (1936), as mentioned above, found swine influenza mouse vaccines ineffective in ferrets when given subcutaneously. There is probably no fundamental discrepancy between his results and ours ; both can be explained in terms of the combined action of two factors—virus titre and animal source of virus.

(iii) *Route of Inoculation.*

Shope (1936) found that the unfavourable effect of using vaccine made from a heterologous species was observed only when subcutaneous inoculations were used, but " the heterologous virus immunized almost as well as the homologous when given intraperitoneally ". He did not find that the intraperitoneal route was notably preferable when homologous vaccines were being used. In our experience, however, intraperitoneal inoculation has always produced better immunity even when one is using mouse vaccine with which to immunize mice ; Tables I and IV afford illustrations of this. We feel once more, therefore, that the unfavourable results of certain vaccinating experiments may be explained simply by the combined influences of several factors, such as a less favourable route of inoculation and a foreign source of virus.

(iv) *State of the Virus.*

We found, in experiments reported earlier (Andrewes and Smith, 1937), that mouse-virus filtrates inactivated by formaldehyde immunized mice almost as well as did living virus ; it was, however, suspected that the inactivation

TABLE II.—*Ferret vs. Mouse Vaccine for Immunizing Ferrets.*

Ferret No.	Vaccine (S.C.).	*Antibodies in serum.	Result of immunity test.			
			Symptoms.	Fever.	Lesions post-mortem.	
					Turbinates.	Lungs.
14	Live ferret virus 5 per cent.	S/25+	+	+	+	−
15	,, ,, ,,	S/25−	+	++	++	−
16	,, ,, ,,	S/5	+	++	+	−
17	,, ,, 0·5 per cent.	S/25	+	+	++	−
18	,, ,, ,,	S/25 or <	++	+	++	−
20	Formol ferret virus 5 per cent.	<S/25	+	++	+	−
21	,, ,, ,,	S/25	++	++	++	−
22	,, ,, 0·5 per cent.	<S/25	++	+	++	++
23	,, ,, ,,	S/125	++	++	++	−
24	Live mouse virus 5 per cent.	*S*−	++	++	++	−
25	,, ,, ,,	S+	−	−	−	−
26	,, ,, ,,	S−	++	+	++	−
27	,, ,, 0·5 per cent.	S or >	± ?	−	−	−
28	,, ,, ,,	S/25 or <	++	++	++	+++
29	,, ,, ,,	<S/5	+	++	++	++
30	Formol mouse virus 5 per cent.	S/5+	+++	++	++	+
31	,, ,, ,,	>S/25	+++	++	++	++
41	*Nil* (controls)	..	+++	+++	++	++
42	,,	..	+++	+++	++	++
43	,,	..	Died	+++	++	+++

+ signs in the last four columns indicate varying degrees of severity of symptoms, height of fever, engorgement and necrosis of nasal mucosa and lung consolidation.

* The titres are expressed in terms of the hyperimmune horse-serum IH_2: thus S/5 implies a serum with 1/5 the potency of the standard serum.

diminished antigenic potency to some extent, for "it chanced in all these experiments that the virus used to prepare the formolized vaccine had a titre rather higher than that of the comparable living virus." The suspicion has been confirmed in later experiments in which inactivation of virus either by formaldehyde or by heat has always reduced antigenic value to some extent. From our recent experiments it appears that virus inactivated by heat makes a vaccine about as good as one made with formaldehyde; Fairbrother and Hoyle (1937) have reported similar results.

Inactivation of virus seems particularly disadvantageous when one is immunizing a heterologous species. Table II shows that living mouse virus will protect ferrets against lung lesions quite as well as will living ferret virus, that formolized ferret virus will also immunize, but that formolized mouse virus produces almost no resistance. In another experiment inactivated ferret virus failed to protect the lungs of ferrets completely; 5 out of 6 ferrets so treated did, however, develop only trifling lung involvement, though tested with a very potent virus. Of 6 ferrets which had received inactivated mouse-virus, on the other hand, 5 had extensive lung involvement as severe as that of the 2 controls, and 2 of them died. Curiously enough, one of these ferrets which showed no resistance and a similar animal (ferret 30, Table II) injected with formolized mouse virus had quite good neutralizing antibodies (S/5) in blood obtained by heart-puncture at the time of the immunity test. We had not previously observed in ferrets a good antibody-titre in the absence of any signs of resistance to the virus; the significance of the observation is not yet apparent.

Living tissue-culture virus having a titre of 10^5 will produce some active immunity in mice when given intraperitoneally in a dilution of 1 : 10. In each of two experiments, however, we found that 1 : 2 tissue-culture virus which had been inactivated by heat did not save a single mouse. In the experiment shown in Table III we endeavoured to eliminate the factor of foreign protein by comparing against each other (1) influenza mouse-lung filtrate mixed with an equal volume of uninoculated tissue-culture, and (ii) influenza tissue-culture virus mixed with an equal volume of filtrate of normal mouse lung. The first mixture was, perhaps, ten times as effective as the second, as might be expected from its tenfold higher titre. We will shortly consider the failure of the heated mouse vaccine to immunize when injected along with chicken tissue-extracts.

EFFECT OF THE ADDITION OF FOREIGN TISSUE-EXTRACTS TO HOMOLOGOUS VACCINES.

Laidlaw and Dunkin (1928) ascribed the inferior response to a heterologous dog-distemper vaccine to the fact that "the antibody mechanism of the recipient of the vaccine is clogged by the multiplicity of antigens". Traub (1938) obtained evidence supporting this view in his experiments with lymphocytic chorio-meningitis; the addition of normal mouse-tissue extracts to a guinea-pig vaccine materially reduced its effectiveness as an immunizing agent for guinea-pigs. Shope (1936), on the other hand, found that the addition of 10 per cent.

TABLE III.—*Mouse vs. Tissue-culture Vaccine for Immunizing Mice. Intraperitoneal Route.*

Nature of vaccine.	Titre of virus.	Result of immunity test.			
		No. of mice in group.	No. dying of influenza.	No. of survivors.	
				With lung lesions.	Without lung lesions.
Live mouse virus : uninoculated tissue culture, 1 : 9	10^5 and 10^4	12	0	6	6
,, ,, ,, 1 : 99	10^4 and 10^3	10	5	4	1
Heated mouse virus : uninoculated tissue culture, 1 : 1	10^6 and 10^5	12	10	2	0
,, ,, ,, 1 : 9	10^5 and 10^4	10	9	1	0
Live tissue culture virus : normal lung filtrate, 1 : 1	10^5 and 10^5	11	4	4	3
,, ,, ,, 1 : 9	10^4 and 10^4	12	5	2	5
Heated tissue culture virus : normal lung filtrate, 1 : 1	10^5 and 10^5	12	12	0	0
Nil (controls)	..	12	11	1	0

TABLE IV.—*Immunization of Mice : Effect of Adding Ferret Extracts to Vaccines.*

Nature of vaccine.	Titre of virus in vaccine.	Route of inoculation.	Results of immunity test.			
			No. of mice in group.	No. dying of influenza.	No. of survivors.	
					With lung lesions.	Without lung lesions.
Mouse virus filtrate undiluted	10^5 and 10^5	s.c.	12	4	4	4
,, ,, 1 in 10	10^4 and 10^4	s.c.	12	9	2	1
,, ,, 1 in 100	10^3 and 10^3	s.c.	11	9	2	0
,, ,, undiluted	10^5 and 10^5	i.p.	12	1	4	7
,, ,, 1 in 10	10^4 and 10^4	i.p.	12	1	4	7
,, ,, 1 in 100	10^3 and 10^3	i.p.	12	10	2	0
,, + ferret protein undiluted	10^5 and 10^5	s.c.	12	11	1	0
,, ,, 1 in 10	10^4 and 10^4	s.c.	12	12	0	0
,, + ferret protein undiluted	10^5 and 10^5	i.p.	12	5	2	5
,, ,, 1 in 10	10^4 and 10^4	i.p.	12	5	6	1
Nil (controls)	..	..	12	10	1	1

swine-serum to a mouse-vaccine did not interfere with its ability to protect mice against swine influenza virus. Our experiments with human influenza virus confirm those of Traub. Mouse-virus filtrates were mixed either with broth or with a filtrate of a 5 per cent. suspension of normal ferret lung. Table IV shows that when the intraperitoneal route was used, the vaccinating power of the mouse-virus was depressed in the presence of ferret tissue-extracts. Subcutaneous vaccines gave a poorer response in any case; the addition of ferret extracts abolished the immunizing power altogether. Of three other experiments on the same lines, two gave results similar to the above. In one of them it was also shown that when ferret protein and mouse vaccine were introduced into opposite flanks of the mice there was no interference with immunization (Table V). The effect was thus a local one, as was shown in a similar experiment by Traub (1938). In one experiment in which very high titre (10^6) living mouse-lung vaccine was given by the intraperitoneal route, the interfering effect of ferret extracts was not clearly demonstrated.

The addition of extracts of uninoculated chicken-embryo tissue-cultures to heated or formolized mouse-lung vaccine was found to diminish vaccinating power in three experiments. Several dilutions of vaccine were used, and the effect, though not large, was seen in each of 7 pairs of contrasting groups of mice. When living virus vaccines were used, however, the interfering effect of tissue-culture extracts was inconstant.

Extracts of normal mouse tissues exercised some adverse influence on immunization of ferrets with ferret vaccines. Thus, of four ferrets immunized with two doses of ferret virus diluted with extract of normal ferret lung, only one developed trifling lung lesions when tested for immunity. On the other hand, three out of four ferrets receiving vaccine diluted with mouse-lung extracts showed lung lesions at autopsy after a similar test. Antibody production was also rather poorer in the ferrets receiving the mouse extracts. The differences observed were, however, less striking than in the experiments with mice.

DISCUSSION.

One can probably explain most of the results of our immunization experiments in ferrets and mice in terms of the combined influence of the four main factors we have reviewed—the virus titre, the virus source, the route of inoculation, and the state of the virus. It is, however, less easy to account for the fact that origin in a different species is particularly detrimental when one is dealing with inactivated viruses. Why, when living mouse virus immunizes ferrets as well as does living ferret virus, is formolized mouse virus useless for ferrets although effective in mice? Why, when living tissue-culture virus is only a little poorer than living mouse virus, should the heated culture virus do nothing? When one is attempting to immunize an animal with living virus which is able to multiply in the tissues of that animal, as in protective inoculation of human beings against smallpox or yellow fever, the fact that the virus comes from a foreign species is immaterial. Can the smaller influence of the source of virus upon active as compared with killed influenza virus be due to the fact that influenza virus does, after all, multiply when

TABLE V.—*Evidence that Interfering Effect of Foreign Protein is a Local one. Subcutaneous Route.*

Nature of vaccine.	Titre of virus in vaccine.	Results of immunity test.			
		No. of mice in group.	No. dying of influenza.	No. of survivors.	
				With lung lesions.	Without lung lesions.
Mouse virus filtrate	10^6 and 10^6	12	7	3	2
,, ,, + ferret extract (mixed)	10^6 and 10^6	12	12	0	0
,, ,, and ferret extract (in opposite flanks)	10^6 and 10^6	12	6	6	0
Nil (controls)	..	12	12	0	0

TABLE VI.—*Reinforcement of Immunity in Ferrets with Live and Formolized Mouse Vaccines.*

No. of ferret.	Interval between last infection and reinforcing vaccine dose.	Nature of vaccine.	Antibody increase after vaccine.	Results of immunity test.			
				Symptoms.	Fever.	Lesions post-mortem in—	
						Turbinates.	Lungs.
HF 7	20 months	Live mouse virus	×125	−	−	−	−
HF 25	20 ,,	,, ,,	×5	±	±	−	−
HF 1	19 ,,	Formol mouse virus	×25	−	−	−	−
HF 30	21 ,,	,, ,,	×25	−	−	±	±
HF 9	26 ,,	*Nil* (control)	..	++	++	+++	−
HF 16	17 ,,	,,	−	±	+	++	±
85	− (fresh ferret)	,,	−	++	++	++	−
86	,,	,,	−	++	++	+++	++

Explanation of + signs as in Table II.

inoculated subcutaneously or intraperitoneally into mice and ferrets ? The evidence obtained hitherto (*cf.* Shope, 1936 ; Andrewes and Smith, 1937) is all against the view that immunization of animals by subcutaneous or intraperitoneal vaccination is dependent on any such multiplication ; yet, if it occurred, it would explain why in our experiments the source of virus influenced the results relatively little when living virus was used. Rickard and Francis (1938) showed that influenza virus injected into mice intraperitoneally in very large doses could reach the lungs and presumably multiply there ; after subcutaneous inoculation, however, it did not reach the lungs, and was recoverable locally and from the regional lymph-nodes in quantities which rapidly diminished in the course of two days. Our own results confirm this. Amongst ferrets also an occasional animal shows a little fever following subcutaneous inoculation with influenza virus ; on one occasion only have we recovered virus from the nose of such a ferret, even though the site of inoculation had been sealed with collodion to prevent transfer of virus therefrom to the ferret's nose. On the whole, it still seems probable that the virus in living influenza vaccines does not multiply under ordinary conditions when given subcutaneously or intraperitoneally, and that any superiority of living over killed virus is not to be ascribed to such multiplication. The results of the present studies, however, incline us to keep an open mind on the matter.

Our results, with those of Traub (1938), strongly support Laidlaw and Dunkin's theory as to the cause of the failure of heterologous vaccine : that the antibody mechanism is so swamped by the large amount of foreign protein presented to it that it cannot deal so efficiently with the minute amount of virus it receives at the same time. Hartley (1935*a*) noted that the antigenic effect of toxin-antitoxin floccules in guinea-pigs was interfered with by the presence of muscle extracts and peptone, themselves non-antigenic. It must, therefore, not be assumed that the interfering substance in heterologous vaccines is necessarily an antigenic protein. We obtained inconclusive answers in two attempts to show whether the active substance in extracts of foreign tissues would withstand boiling.

Hartley (1935*b*) also showed that a given quantity of diphtheria formol-toxoid immunized guinea-pigs better when it was contained in a large amount of diluent. He suggested that " when the antigen is presented in the larger volume, a greater number of antitoxin-producing cells are engaged and stimulated ". This observation may give a clue to the particularly large effect of the foreign extracts on inactive vaccines. The effect of the foreign substances has been shown to be a local one. Viruses are almost certainly aggregated by the processes of inactivation : we have found, for instance, that a mouse lung filtrate, passed through a 0·7 μ gradocol membrane and then formolized, loses much of its antigenic power if it is refiltered through a membrane of the same size after formolization. Virus thus aggregated may diffuse less readily in the tissues, and so be more influenced by the local effects of the foreign tissue extracts.

One result of our studies must be emphasized : neither in ferrets nor mice have we induced active immunity by means of *inactivated heterologous* vaccines. This result is not encouraging for the use of inactivated virus in man, in whom heterologous vaccines must necessarily be used. It is true that inactivated

mouse vaccines will produce a substantial rise of neutralizing antibodies for influenza virus in human beings; but it might be argued that this may correspond to the reaction of the two ferrets discussed earlier, in which a rise of antibody, but no resistance, was apparently induced by vaccination. One grain of comfort, however, remains; ferrets with a waned immunity can have that immunity restored by mouse vaccine, and as effectively by the formolized as by the living mouse virus (Table VI). We have suggested previously that the immunity of most human beings may be comparable with that of ferrets having such basic immunity.

CONCLUSIONS.

Vaccines made from infected ferrets' tissues have proved useless for immunizing mice against influenza virus, though mouse-tissue vaccine is highly effective.

On the other hand, living influenza vaccines are of equal value for immunizing ferrets whether made from ferret or mouse tissues. Analysis of these phenomena appears to show that the efficacy of a vaccine depends upon the resultant of several factors: (i) the titre of the virus used for preparing the vaccine, (ii) the animal source of the vaccine, (iii) the route of inoculation, and (iv) the state of the virus, whether living or inactivated. The relative poorness of vaccines made from heterologous species is probably accounted for by the fact that foreign tissue extracts interfere with the immunizing response. Some evidence suggests that this interfering effect is greater when inactivated vaccines are being used.

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EXPERIMENTAL ACUTE PERCHLORIDE INTOXICATION.

GEORGE L. MONTGOMERY.*

From the Royal Hospital for Sick Children, Glasgow.

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THE original purpose of the experiments which form the basis of this short communication was to determine the nature and distribution of the earliest renal changes following the intravenous injection of mercuric chloride in a series of rabbits, and to correlate these findings with any alterations in their blood chemistry. The investigation failed to demonstrate unequivocal renal damage by histological methods (including mitochondrial preparations) within two hours of the injection of the perchloride, but certain changes in the blood chemistry occurred within that period. Mercuric chloride differs from other substances used experimentally to damage the renal tissue in that its exhibition produces some degree of shock or collapse in the animal. For example, the immediate effect of intravenous doses of 2·5 mg. $HgCl_2$ per kilo body-weight (lethal in five to seven days) was that the animals remained stunned and motionless for some minutes after the injection and sat listlessly in their cages for a variable period up to one hour, after which they moved about normally but refused food and water until death. The immediate slowing of the peripheral circulation was shown by collapse of the ear veins, so that after the injection it was sometimes a matter of great difficulty to obtain blood for examination. These manifestations of perchloride intoxication were exaggerated if larger doses were employed, when colitis and anuria frequently developed: care was taken to avoid such effects in this series of experiments. In general, although the blood non-protein nitrogen and chlorides underwent minor modifications, the most outstanding immediate effect of the intravenous injection of small doses of perchloride of mercury in rabbits was a rapid fall in the blood-serum protein with proteinuria, accompanied by evident circulatory collapse. Peters and Van Slyke (1931) state that a fall in blood-serum protein is usual in traumatic shock, although in that instance it is not as a rule accompanied by proteinuria. Mercuric chloride intoxication, however, affords an example of a chemical substance producing circulatory collapse with proteinuria and a fall in serum protein, and its use experimentally in small doses over short periods of time gave an opportunity of studying the relationship of these factors. On account of the frequency with which clinical records contain references to the use of saline transfusions to combat the shock syndrome in perchloride poisoning, some additional observations were made on the effect of intravenous saline.

* Gardiner Lecturer in the Pathology of Diseases of Infancy and Childhood, University of Glasgow.

METHODS.

Thirty-two animals were used, all healthy adults whose urines were free from abnormal constituents. The general procedure was to bleed the animals and then inject into an ear-vein an aqueous solution of $HgCl_2$, the dose being 1 c.c. of an 0·25 per cent. solution (=2·5 mg. $HgCl_2$) per kilo body-weight. This amount was lethal in five to seven days and produced circulatory collapse in the animals, but was insufficient to induce anuria or diarrhœa. The rabbits were killed by breaking their necks at intervals varying from 15 minutes to 2 hours after the injection of perchloride, blood being withdrawn instantly from the right ventricle of the heart: in this manner blood and renal tissue were obtained simultaneously without the delay incurred by bleeding collapsed ear-veins. Other animals were allowed to live for 2 hours and bled 30, 60 and 120 minutes after the injections, thus forming a control series to those killed within that period, and showing that there was no significant difference between blood from the ear and blood from the cardiac ventricle. The amount of blood withdrawn from the ear-veins and from the heart was always 2½–3 c.c., and on these specimens blood urea concentration, non-protein nitrogen, whole blood and plasma chlorides were estimated by standard methods, R.B.C. and plasma volumes by the hæmatocrit; since only small quantities of blood were available the refractometer method was employed for serum protein.

In the saline experiments, three control rabbits were bled, and then given intravenous injections of sterile saline at blood-heat in quantities varying from 25–50 c.c., the injection occupying 2 minutes and the rate kept as constant as possible. As before, blood was taken for examination 30, 60 and 120 minutes thereafter. Two weeks later the same animals were given their appropriate dose of perchloride intravenously, followed immediately, without withdrawing the needle from the vein, by the exact dose of saline given previously. These animals also were bled at 30- and 60-minute intervals and killed after 2 hours, blood being obtained from the heart as in our first series. Five rabbits other than the controls were given saline and mercury in the same manner.

To determine the relationship of proteinuria to the fall in serum protein a group of eight male rabbits was used. Before the experiments their bladders were drained by catheterization and the urine found free from abnormal constituents; the animals were bled and intravenous perchloride administered, after which they were placed in hopper cages devised to collect all the urine and exclude fæcal contamination. Actually most of them did not pass urine during the experiment, and their bladders were emptied by syringe immediately after death. Blood-sampling was carried out as described above, and the serum protein and urinary protein values estimated.

RESULTS.

Blood-urea and blood non-protein nitrogen.

Intravenous $HgCl_2$ alone or when followed by normal saline produced constantly a slight and relatively unimportant increase in the blood-urea and non-protein nitrogen concentrations during the 2 hours, the average for the former being 10·8 mg. and for the latter 4·3 mg. per 100 c.c.

Serum protein and proteinuria.

In four of the forty rabbits the blood-serum protein level was reduced by less than 0·2 g. per 100 c.c., and only traces of protein could be detected qualitatively in the urines of these animals. In all others the fall in the blood-serum protein was always considerable, averaging 0·85 g., and in three instances exceeding 1 g. per 100 c.c. Proportionately the greatest fall in the serum-protein level occurred between 30 and 60 minutes after the injection of perchloride, proteinuria being marked and demonstrable by ordinary clinical tests as early as 15 minutes after the injection of perchloride. Microscopically, scanty red blood-corpuscles appeared in the urine occasionally within a few minutes of the injection, but granular casts were never detected during the 2-hour period. To determine whether the fall in blood-serum protein could be accounted for by the proteinuria alone, the total urinary protein excreted in the 2-hour period was estimated in a series of eight rabbits whose urines were collected as described above. The plasma volume in each animal had been estimated by the hæmatocrit as a percentage of its blood-volume which, for this experiment, was calculated as 9·6 per cent. of its body-weight—a figure obtained in rabbits by the vital red method by Nice and Katz (1934). This figure was common to all eight rabbits, and on this basis their total plasma volume (which may be taken as identical with the total serum volume) and their total fall in serum protein were assessed, the loss of serum protein being related to the total proteinuria with this result :

Total fall in blood-serum protein.	*Total urinary protein.*
0·74 g.	1·16 g.
0·66 g.	1·18 g.
0·83 g.	1·3 g.
0·43 g.	0·95 g.
0·27 g.	0·68 g.
0·39 g.	1·0 g.
0·9 g.	1·38 g.
0·67 g.	1·13 g.

The first three of these rabbits were given perchloride alone, and the other five perchloride followed by saline as described above. In the latter, a fall in blood-serum protein averaging 0·15 g. per cent. occurred during the 2 hours due to dilution alone, and this figure was deducted from the total loss of blood-serum protein in each case. The fall in serum-protein was more variable in the animals which received saline, but it is evident that in all eight rabbits the proteinuria exceeded the loss of protein from the blood by a considerable amount.

Chlorides.—After $HgCl_2$ alone, the behaviour of whole blood and plasma chlorides was variable; in some instances these values increased, in others there was a fall, while in some the values were unchanged. On the other hand, while saline alone always increased both the whole blood and plasma chlorides, this increase was not so marked in the former if the saline was preceded by perchloride.

Red blood-cell volume.—This factor remained practically unchanged after perchloride whether or not it was followed by saline.

DISCUSSION.

In rabbits poisoned intravenously with small doses of mercuric chloride, an appreciable reduction of serum protein with proteinuria commenced immediately after the injection, a fall of more than 1 g. per cent. being recorded on several occasions within an hour of the injection in 1·5 kilo rabbits. The ratio of the fall in the blood-serum protein to the protein lost in the urine varies from 1 : 1·5 to 1 : 2, the higher ratios being in the animals which received the saline. These ratios are naturally approximate, as they depend partly on the total serum-volume calculated from the blood-volume assessed by the formula of Nice and Katz, but the essential point is that the excess of protein in the urine was always sufficiently great to be independent of the blood-volume calculation, which, indeed, was common to them all. The source of this protein in the urine must have been the blood-serum, as the lack of microscopic evidence of renal damage excludes the possibility that it was kidney-protein. It is therefore unnecessary to postulate any mechanism other than renal excretion to account for the fall in the blood-protein level, and it is evident that some—probably more than one-third—of the serum protein lost through the kidneys must have been replaced to the blood from the tissue depots. There is little doubt that in prolonged proteinuria such replacement of serum protein from the tissues frequently does take place, but the surprising feature demonstrated by these short 2-hour experiments is the rapidity with which serum protein is lost and replaced. In the experiments described above, the protein loss appeared proportionately greatest in the first 40 minutes, and thus coincided with the most severe stage of shock or collapse of the animal : on the other hand, more than one-third of the protein lost from the blood must have been replaced within the 2 hours of the experiment, and may be related to the amelioration of the shock syndrome during the latter part of the experimental period. Perchloride intoxication differs from traumatic shock in the important fact that in the former protein is actually lost to the body through renal excretion, whereas this has not been demonstrated in the latter, and although the part played by the fall in serum protein in the genesis of shock is far from clearly defined, some relationship may exist between the blood chemistry and the clinical condition.

The fact that it is unnecessary to postulate any mechanism other than renal excretion to account for the fall in the blood-protein level allows the inference that the initial lesion in perchloride intoxication is renal, and this is supported by an examination of the fate of mercuric chloride exhibited experimentally in dogs or accidentally in the human subject. Sollmann and Schreiber (1936) published an account of autopsy material from patients who died of poisoning with bichloride of mercury, and from their analyses the concentration was found to be uniformly highest in the kidneys (average 3·8 mg. per 100 g. of renal tissue). They found the liver to hold one-half to one-third of the kidney concentration, the other tissues considerably less,

and the blood least with one-fortieth of the renal concentration. In their studies of acute mercurial poisoning, these authors have shown that, apart from loss by early emesis, the elimination of mercury by fæces, gastric lavage and urine is extremely small, the latter especially being almost negligible even in mild cases with polyuria. Thus from chemical analyses there is evidence that the renal tissue is primarily affected by absorbed mercuric chloride, but morphological evidence of renal damage is difficult to obtain when only small doses of perchloride are employed over a short period. Using larger doses, Burmeister and McNally (1917) claimed to produce in dogs degenerative tubular changes within 5 minutes of oral administration, and similar results were obtained by Photakis and Nikolaidis (1929) and Koranyi (1934). On the other hand, Moore, Goldstein and Canowitz (1929), in their mitochondrial studies, could only occasionally observe histological changes within 24 hours, and in their experiments positive findings were always associated with urea retention. With regard to the actual site of the lesion, while the older writers regarded $HgCl_2$ as a tubular poison only, Hunter and Roberts (1932) noted fragmentation and rupture of the glomerular basement membranes as a prominent feature of their experiments in dogs, in which they used a dose similar to ours but over a period of 3 days. It is clear that the microscopical changes are all related to dose and time, and in our experiments over 2-hour periods no appearances were seen even in mitochondrial preparations which could not be duplicated in the kidney sections of normal control rabbits. Proteinuria and the occasional presence of scanty red blood-cells and traces of hæmoglobin in the urine may be interpreted as indicating damage to the basement membranes, but sections stained by various modifications of the aniline blue methods failed to reveal any abnormality. In this respect, therefore, it may be said that proteinuria is a more sensitive index of renal damage than ordinary histological methods.

Intravenous saline is a clinical measure for diminishing the intensity of the shock syndrome in perchloride poisoning, but no such effect was noted in our rabbits. It may be that the saline was administered slightly quicker under experimental conditions, and it is certain that proportionately to the blood-volume of the animal a larger dose of saline was given than is the case with human patients. It was noticeable that saline never produced œdema. The rabbits were weighed immediately before each experiment and again just before death, and in animals which did not pass urine during the experimental period, there was an increase in weight which was exactly accounted for by the amount of fluid injected, whereas in animals which did urinate, the weight remained constant. Although water was provided, none of the animals drank. Using considerably larger doses of salt and abundant fluid by mouth, Dunn, Oastler and Tompsett (1934) produced œdema in perchloride rabbits within 2 or 3 days of the injection, but it is not possible to give sufficient fluid intravenously in rabbits to achieve this result. It is possible that a relatively large saline transfusion given within 2 minutes of the injection of perchloride opened up additional glomeruli to the poison, and so led to more diffuse although possibly less intense damage. Unfortunately, no histological evidence could be obtained to this effect, but the proteinuria in the saline experiments was usually greater than that which occurred when perchloride alone was used.

SUMMARY.

The injection of small doses of mercuric chloride intravenously in rabbits produced a very rapid fall in blood-serum protein with proteinuria accompanied by circulatory collapse. These findings were present before any alteration in the tissues could be detected microscopically, but evidence is discussed which suggests that the renal lesion is the primary effect of the poison, with loss of serum protein as its result, which in turn may be related to the shock syndrome.

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THE PURIFICATION OF INSECT-TRANSMITTED PLANT VIRUSES.

F. C. BAWDEN AND N. W. PIRIE.

From the Rothamsted Experimental Station, Harpenden, Herts, and the Biochemical Laboratory, Cambridge.

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In the past few years specific nucleoproteins have been isolated from plants infected separately with a number of different viruses. These nucleoproteins have been obtained in apparently pure forms, and there is no evidence conflicting with the view that they are the viruses themselves. All the viruses that have yet been isolated, namely, tobacco mosaic, potato " X ", cucumber 3, tomato bushy stunt, and tobacco necrosis, have been found to have closely similar analytical compositions, differing significantly only in the quantity of nucleic acid they contain (Bawden and Pirie, 1939). These have widely different properties, both *in vivo* and *in vitro*, but they are all among the more stable viruses, and the list contains no virus known to be transmitted by insects. Therefore, although it is reasonable to expect that some other plant viruses are also nucleoproteins, it would be premature to assume from the results obtained with the five named viruses that all plant viruses are chemically similar.

The viruses that are known to be insect-transmitted can be divided conveniently into two types on the basis of their relationships with their insect vectors. Viruses of the first type are not transmitted by their vectors immediately after a short feeding period on infected plants. A so-called incubation period is necessary before the insects become infective, but once they are able to transmit they continue to do so for long periods, sometimes for the remainder of their lives. Viruses of the second type have no significant incubation period in their vectors, and a few hours after ceasing to feed on infected plants these lose their power of infecting healthy plants. That this difference is determined by the virus and not the vector is shown by the fact that some insects transmit both types of viruses.

In this paper attempts to purify two insect-transmitted viruses of the second type are described. Most of the work has been done with potato virus " Y ", but many preparations of Hyoscyamus virus 3 have also been made. Watson (1936, 1938) has shown that these two viruses are transmitted by *Myzus persicæ* Sulz. and some other aphids in essentially the same manner. One curious feature in their transmission is that the efficiency of the vectors is much reduced by continuous feeding. The optimum conditions for transmission are obtained by using aphids that have been starved for at least 1 hour, allowing these to feed for periods of only a few minutes on the infected plants and transferring them immediately to healthy plants.

These two viruses occur in infective sap in much smaller concentrations than those previously described. As they are also less stable and their properties in many ways approximate more closely to those of some of the normal plant proteins, their purification is more difficult, and it is impossible to make confident statements about either the purity or properties of the preparations. Sufficient evidence has been gained, however, to indicate that these two insect-transmitted viruses do not differ from those previously isolated in the general plan of their chemical constitution.

Preparation of Potato Virus " Y ".

Preparations of potato virus " Y " have all been made from tobacco plants, variety White Burley. The work was done mainly in the winter months, for the yield of virus " Y " is then greater than in the summer ; furthermore, plants then contain smaller amounts of troublesome constituents that interfere with the purification. The plants were cut down about a month after infection, when showing definite dark-green vein-banding symptoms, minced, and the sap expressed through muslin.

Na_2HPO_4 is added to the sap until a copious precipitate is produced, and this is removed by a few minutes' centrifugation at 3500 r.p.m. 300 g. of ammonium sulphate is added to each litre of the clarified sap, together with some NH_4OH to keep the mixture neutral, because much of the virus is lost if the pH falls sufficiently to coagulate the normal plant proteins. After standing for an hour to allow complete flocculation of the virus, the fluid is centrifuged for about half-an-hour, the brown supernatant fluid being discarded. The precipitate is suspended in a measured volume of water (about one-fifth of the volume of the original sap) and centrifuged. One-quarter of a volume of saturated ammonium sulphate solution is added to the supernatant fluid and, after standing for an hour, the mixture is centrifuged. The bulky, greyish-green precipitate is discarded, and the supernatant fluid is two-fifths saturated with ammonium sulphate. After standing for an hour, the fluid is centrifuged and the yellow supernatant fluid discarded.

The precipitate at this stage is a soft brown mass that will not pack tightly in the centrifuge tube. It is suspended in one-fifth of its volume of phosphate buffer at pH 8 and dialysed against running tap-water for from 6 to 8 hours. The dialysed fluid usually has a volume of about one-twentieth of the original sap and a solid content of from 1–2 per cent. If this solution is treated with different amounts of ammonium sulphate between one- and two-fifths of saturation, it can be separated into a number of fractions. Although these have different physical properties, each contains virus, and the process does not lead to any useful fractionation of the virus. Some virus can be prepared directly by high-speed centrifugation of the concentrated dialysed solution, but as it contains many other proteins that also sediment readily, it is more convenient to destroy some of these by incubation with trypsin. Incubation for 6 hours at 37° C. and pH 8 in the presence of 0·4 per cent. " Pangestin " (Difco) removes most of the contaminating proteins without seriously reducing the virus content. Unless the solution is highly buffered

it will be necessary to readjust the pH by the addition of alkali during the incubation. After such incubation, one-quarter of a volume of saturated ammonium sulphate solution is added and, after standing for 2–3 hours at 0° C., the mixture is centrifuged. Two-fifths of a volume of saturated ammonium sulphate solution is added to the supernatant fluid, and after standing for an hour the mixture is centrifuged for an hour at 3500 r.p.m. to pack the precipitate tightly. The precipitate is suspended in a volume of water equal to one-hundredth of that of the original sap, the pH adjusted to pH 7·5, and the fluid centrifuged for a few minutes to get rid of insoluble materials. The final supernatant fluid is then centrifuged at 15,000 r.p.m.

For unknown reasons it has never proved possible, at this stage, to sediment all the virus from a solution by centrifuging at 15,000 r.p.m. in a rotor of 8 cm. radius. About 80 per cent. is sedimented in from 3 to 4 hours and some of the remainder can be sedimented by a further period of centrifugation, but some always remains in the supernatant fluid. The pellets resulting from the high-speed centrifugation are usually opaque, showing only the faintest trace of birefringence at their edges. Occasionally, however, they are pale green and almost clear. The material constituting the final virus preparation is merely a minor constituent of these pellets. The nature of the major constituent varies somewhat in different preparations. Most often it is either a tough brown material that is suspended in water with difficulty, or a pale greenish-grey material that is suspended readily. Some of the properties of the second material, which sediments from neutral solutions much more readily than the virus, will be described later. The pellets are suspended in a volume of $M/20$ NaCl solution equal to about one-tenth of that from which they were sedimented, and then centrifuged for an hour at 10,000 r.p.m. Many of the contaminants sediment with this treatment, whereas most of the virus remains in the supernatant fluid, which now, for the first time, shows definite anisotropy of flow if shaken between crossed polarizing screens.

The process of differential centrifugation, that is, 3 to 4 hours at 15,000 r.p.m., followed by shorter periods at 10,000 r.p.m. on the resuspended pellets, is continued until a 0·3 per cent. solution is colourless, and the pellet obtained by centrifuging at 10,000 r.p.m. is small, homogeneous and birefringent. All the pellets separating at the lower speed contain some virus. They are therefore mixed, extracted with $M/20$ NaCl solution at pH 7·5, and the process of differential centrifugation repeated to recover the virus. By this method about one-half of the virus, estimated by serological titres, that was present in a preparation before incubation with trypsin can be obtained in a colourless state showing strong anisotropy of flow. The yields obtained have varied between 0·5 mg. and 1·5 mg. per litre of infective sap. This contrasts widely with the 2 g. or more virus that can be isolated from a litre of sap from plants infected with tobacco mosaic virus. As tobacco mosaic virus is the commonest contaminant in tobacco plants, all the anisotropic preparations of potato virus "Y" were tested for its presence, both serologically and by inoculation to *Nicotiana glutinosa*. This was necessary, for if one plant out of the 200 or so used for each preparation became infected, even before symptoms of tobacco mosaic became obvious on it, the material isolated would consist largely of tobacco mosaic virus.

Properties.

In describing preparations of other viruses that we have made it was possible to show by various tests that they appeared to be homogeneous. No similar claim can be made for the preparations of potato virus " Y ", for too little material has been available for rigorous testing. In their general physical properties, however, the preparations of this virus resemble those of the other anisotropic viruses, especially those of potato virus " X ", although they are more readily denatured by heat, acid and ageing. The intensity of anisotropy of flow that they show is similar to that shown by preparations of potato virus " X ", *i. e.* it is clearly visible when a 0·02 per cent. solution in a tube of 1 cm. diameter is shaken in a polarizing box. The liquid crystalline pellets prepared by the high-speed centrifugation of solutions of viruses " X " and " Y " also resemble one another closely, and the two viruses precipitate in a similar manner with acid and ammonium sulphate. When completely precipitated the material is difficult to see and appears amorphous under the microscope. But with insufficient acid or salt (about pH 5 or one-tenth saturation with ammonium sulphate) for complete precipitation the fluids develop a slight sheen and show increased anisotropy of flow. The precipitates produced from highly purified preparations of virus " Y " with acid and salt dissolve with difficulty, although with cruder preparations the precipitation is readily reversible. Potato virus " Y " sediments from neutral solution rather more slowly than potato virus " X ". No differences have been found between the two to suggest why one should be so readily transmitted by aphids and the other not.

The activity of purified preparations of virus " Y " is considerably less than that of preparations of the other anisotropic viruses previously isolated, and it seems probable that the purification processes inactivate much of the virus. Activity has been tested both by serological and infectivity tests. Attempts to produce antisera in rabbits by the intraperitoneal injection of crude infective sap failed, but good precipitating antisera were obtained by two intravenous injections of 2 mg. of a partially purified preparation. The virus-antiserum precipitate has a fluffy, open structure, similar to that given by the other anisotropic viruses and by bacterial flagellar antigens (Bawden and Pirie, 1938*b*). The serological titres of the purified preparations, *i. e.* the greatest dilution at which 1 c.c. gives a visible precipitate when mixed with1 c.c. of diluted antiserum, have varied between $1 : 5 \times 10^5$ to $1 : 10^6$. These titres are approximately one-sixth of those obtained with carefully purified preparations of potato virus " X ". Some of this difference results from the different method used for testing virus " Y ". It is rapidly inactivated at temperatures around 55° C., and the tests could not, therefore, be carried out for 12 hours at 50° C. Instead, the tubes containing the virus-antiserum mixtures were placed in the water-bath at 50° C. for a few minutes at hourly intervals to ensure complete mixing by convection currents, and were kept for the remainder of the time at 1° C. Readings were made after 24 hours. With preparations of potato virus " X " this method was found to give titres about half as great as those obtained by the method previously used. Even when an allowance

is made for methods of testing, therefore, the serological activity of the virus "Y" preparations is only about one-third of that of virus "X" preparations.

The weight of a purified preparation required to cause infection when rubbed on to tobacco plants has varied from 10^{-5} to 10^{-7} g., that is, the infection end-point is little or no greater than the precipitation end-point. In the clarified untouched sap this is not so, for the precipitation end-point of this varies with different samples from about 1/4 to 1/12, whereas the infection end-point is usually greater than 1/10,000. The ratio of infectivity to serological activity, therefore, is greatly reduced by the purification methods. As the virus does not give countable local lesions, accurate quantitative infection tests could not be made, but the reduction in the ratio seems too great to be explained by aggregation as suggested for tobacco mosaic virus (Bawden and Pirie, 1937). It seems likely that during the isolation many of the virus particles undergo slight changes that render them non-infective but do not destroy their serological activity. Loring (1938) has suggested that salts do this to potato virus "X", and it has been found possible to bring about changes of this type in all the other viruses that have been studied by treatment with agents like formaldehyde, nitrous acid, hydrogen peroxide, ultra-violet light and X-rays.

Occasionally the whole, or the greater part, of a preparation has appeared finally as a serologically inactive and non-infective product that shows strong anisotropy of flow but is insoluble, that is to say, it sediments in an hour when centrifuged at 2500 r.p.m.. Insufficient material of this type has been available for a detailed study, but its content of phosphorus and carbohydrate is similar to that of the soluble preparations. An explanation of this cannot be given, but the insoluble material may be analogous to the anisotropic precipitates given by tobacco mosaic virus and potato virus "X" with clupein and related materials ; or it may be simply an extreme case of the aggregation that is characteristic of the anisotropic viruses that have been salted out.

Provided that the purified preparations of virus "Y" are kept cold they remain active and show anisotropy of flow for some weeks, but at room temperature they become inactive and lose their optical properties in a few days. If crude sap is acidified to below pH 5 the virus is lost. By contrast, exposure for 3 hours to pH 5 at 15° C. had no effect on the activity of a purified preparation, and after 3 hours at pH 4 the activity was only reduced by one-half. After 3 hours at pH 9·2 a preparation was non-infective but still retained one-half of its serological activity. After this time at pH 10·3 both infectivity and serological activity were destroyed.

Purified preparations of virus "Y" are not inactivated by freezing and thawing. Thus they differ from those of tomato bushy stunt virus, but resemble those of the other anisotropic viruses that have been purified. As many of the normal plant proteins are irreversibly coagulated by this treatment, it might be of use in the purification. However, it has been found that this virus is readily absorbed on to surfaces, and procedures separating the virus as a precipitate, *e. g.* centrifugation, lead to much smaller losses than those in which contaminants are precipitated from fluids containing the virus. During the course of preparation the whole of the virus has occasionally disappeared,

a loss attributed to the absorption of virus either on to the precipitate separating with one-fifth saturation with ammonium sulphate, or on the precipitate produced by the ageing of the normal sap constituents. This is in agreement with the observation that virus " Y " is inactivated at greater pH values in crude preparations than when purified, for normal sap contains constituents precipitated at about pH 5. Virus " Y " is inactivated by absorption on to charcoal, but not by kieselguhr, talc or kaolin. Attempts have been made to use these materials in the purification methods. They have not proved as useful as the methods described above, but it is possible to separate the virus by centrifuging suspensions of kieselguhr in partially fractionated preparations for a few hours in the closed rotor of a Sharples centrifuge. The greater part of the virus is held by the thin layer of sedimented kieselguhr while the rotor is being emptied, from which it can easily be extracted in a small volume of water.

The purified preparations of potato virus " Y " contain from 14–16 per cent. of nitrogen—a value inside the range usually found for proteins. The most usual impurity is a complex containing both lipoid and carbohydrate. This contains from 6–10 per cent. of nitrogen, and its presence, therefore, leads to low nitrogen figures. Total carbohydrate estimations have been carried out by the orcin method (Pirie, 1936) used for other viruses, but with virus " Y " the results are often less certain because of the turbidity of the reaction mixture. This turbidity probably results largely from the presence of the contaminating lipo-protein, for this alone gives an intense turbidity. The virus preparations most active serologically have shown the least turbidity, and have given a colour corresponding to 3 per cent. of carbohydrate, estimated as glucose. As with potato virus " X ", it is often difficult to reduce the carbohydrate content to below 5 per cent. The phosphorus content is from 0·3–0·5 per cent. and is a fairly reliable criterion of virus activity, for the other proteins present in partially purified preparations contain no phosphorus and the contaminating lipoid-protein contains little. Too little material has been available for attempts to isolate a nucleic acid from this virus, but from its general behaviour it seems probable that this preparation consists essentially of a nucleoprotein more similar to tobacco mosaic virus and potato virus " X " in its content of nucleic acid than it is to tomato bushy stunt or tobacco necrosis viruses.

Absorption spectra.

It is difficult to get optically-clear solutions even at dilutions as low as 0·02 per cent., for during the necessary centrifugation there is a serious loss of virus. The interpretation of the absorption spectrum, therefore, is rather more difficult than with the viruses previously studied. Tobacco mosaic, tomato bushy stunt and tobacco necrosis viruses all have spectra of the same type, *i. e.* there is a maximum at 260 mμ., followed by a minimum at 245 mμ., and at shorter wave-lengths the absorption becomes intense. With potato virus " Y ", by contrast, although the absorption at 260 mμ. (the region associated with absorption by nucleic acid and other purine-containing substances) is as strong as that with solutions of tobacco mosaic virus of the same concentration,

there is no minimum at 245 mμ. This effect may result from the summation of an absorption curve similar to that found for the other viruses with another curve produced partly by another constituent with an absorption maximum at about 245 mμ., and partly by the light scattered by the small amount of suspended material. If, as in other systems, the scattering is proportional to $1/(\text{wave-length})^4$, scattering alone could not explain the phenomenon. A similar effect has also been obtained with both strains S and L of potato virus "X". Preparations of these were made by the methods previously described (Bawden and Pirie, 1938*a*). They were as clear as protein solutions ever are, yet they showed a similar anomalous absorption at 245 mμ. An attempt to explain this phenomenon will be made later when more material is available.

The lipoid-containing contaminant.

In preparations made during the spring and summer the most troublesome contaminant has been a grey, or sometimes greenish material that is not precipitated with one-fifth saturated ammonium sulphate solution but is precipitated at two-fifths of saturation. It can be readily differentiated from the virus, as it is much more easily sedimented from neutral solutions by high-speed centrifugation and is apparently unaffected by boiling. Solutions of this material do not show anisotropy of flow when agitated in polarized light, but they are opalescent and often show a pronounced shimmer in ordinary light, similar to that shown by suspensions of blood-corpuscles. The material does not react with virus-antiserum.

When solutions of the contaminant are dried frozen they give a material with a light, open structure, from which 20 per cent. to 40 per cent. by weight can be extracted with lipoid solvents such as benzene. The extracted lipoid is partly crystalline when solutions in benzene or alcohol are evaporated, and it usually contains 0·1–0·2 per cent. of phosphorus; as would be expected, the phosphorus can be concentrated into the acetone-insoluble fraction. After removal of the fat, the residue contains all the nitrogen and carbohydrate of the original material; its nitrogen content is from 9–14 per cent., and the carbohydrate is much more variable but is usually between 10 and 20 per cent. of the defatted residue.

The nature of this material is unknown. It may be a specific product of virus "Y" activity, for no such material has been found either in healthy plants or in plants infected with other viruses. The material is reversibly precipitated with acid and ammonium sulphate and is heat-stable, so that none of the treatments used in preparing the other viruses would be expected to destroy it. However, extracts of plants infected with other viruses have not been re-investigated since this material has been recognized in preparations of potato virus "Y". If present in them it is certainly removed from the viruses with greater ease than from virus "Y", and this removal is accompanied by less serious losses of virus. But the quantitative differences in virus-content of sap from plants infected with virus "Y" and with the viruses previously investigated are so great that this contaminant, if present in other infective

extracts, would be only a minor constituent of a virus preparation and so might remain unnoticed.

Preparation of Hyoscyamus Virus 3.

The work on Hyoscyamus virus 3 was done mainly in the summer, for infected plants are so crippled in the winter that they yield little sap. Using similar methods to those for potato virus " Y ", material showing anisotropy of flow has been isolated with properties essentially similar to those of virus " Y ". The yields have been about 1–3 mg. per litre of sap, but the yield per plant has been less than with virus " Y " because of the greater reduction in growth caused. Although these two viruses have such similar properties and are transmitted in the same way, they are not related strains, for antisera prepared against one does not react with the other.

SUMMARY.

Liquid crystalline preparations of nucleoproteins have been made from the sap of tobacco plants infected with the two insect-transmitted viruses, potato virus " Y " and Hyoscyamus virus 3. The yields are from 0·5 to 3·0 mg. per litre of infective sap. These proteins give specific precipitates, of the flagellar type, with antisera when diluted to $1 : 10^6$. They are infective, but the methods used in the purification processes seem to destroy the infectivity of much of the virus without destroying its serological activity. The chemical and physical properties of preparations of these viruses closely resemble those of potato virus " X ". Some properties of a lipoid-containing contaminant are described.

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PANTOTHENIC ACID AND THE GROWTH OF *STREPTOCOCCUS HÆMOLYTICUS*.

HENRY McILWAIN.*

From the Department of Bacterial Chemistry (Medical Research Council), Bland Sutton Institute of Pathology, and from the Courtauld Institute of Biochemistry, Middlesex Hospital, London, W. 1.

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In a previous paper (McIlwain, Fildes, Gladstone and Knight, 1939) it was shown that certain hæmolytic streptococci would grow on a mixture of which all ingredients were chemically defined except the constituents of Bacto-peptone. Subsequently it was found that no growth took place when the peptone was replaced by acid hydrolysed casein. The present work indicates that the necessary substance in Bacto-peptone which is absent from hydrolysed casein is probably pantothenic acid, which was first characterized by Williams (1933) as a nutrient for yeast.

BACTERIOLOGICAL TECHNIQUE.

The procedure was that used by McIlwain *et al.* (1939), except that glutamine was added in a concentration of $M/2000$ in place of the then unknown sample to be tested and that hydrolysed casein replaced the Bacto-peptone. Preparations of the present unknown were tested on this inadequate medium in five-fold dilutions. The test coccus was the well-known "Richards" strain, passaged in mice every month and maintained on blood-broth. Suspensions were made from 20-hour agar slopes and diluted in the casein mixture, approximately 1000 cocci being added per tube.

Preparation of the hydrolysed casein.—A mixture of hydrochloric acid (170 ml.) and water (110 ml.) was stirred quickly with commercial casein (200 g.) and the mixture autoclaved at 120° C. for ¾ hour. The resulting solution was neutralized, while cooling, with 10*N* NaOH (*ca.* 180 ml.) and filtered through a bed of paper-pulp on a Buchner funnel. It contained the calcium of the original casein (0·272 per cent.), which was removed by boiling with a slight excess of oxalic acid and filtering. This hydrolysate was added in a concentration corresponding to 1 per cent. casein, in place of the Bacto-peptone of McIlwain *et al.* (1939).

PARTIAL ISOLATION OF THE ACTIVE MATERIAL.

A preliminary survey indicated yeast to be the most readily available source containing workable quantities of the present factor. A hot extract of yeast lost only a little of its activity on autoclaving at 120° C. for 20 min., and

* Leverhulme Research Fellow.

none on heating at 100° C. in neutral solution for 1 hour. Heating for 3 hours at 100° C. in *N* H_2SO_4 caused considerable loss of activity, and in *N* NaOH, complete loss. Active material was not precipitated by lead or mercury salts nor by phosphotungstic acid. No appreciable adsorption occurred with norite or fuller's earth in neutral or acid solutions. Activity was not extracted from a neutral solution by amyl alcohol but slow extraction occurred from acid solutions.

Preparation of yeast extract.—The sodium sulphate method of Deutsch, Eggleton and Eggleton (1938) was used. Brewers' yeast (6 kg.; loss on heating at 100° C., 75 per cent.) was crumbled, thoroughly mixed with anhydrous sodium sulphate (2·5 kg.) and heated to 35° C., when it became liquid. Paper pulp (160 g.) was uniformly mixed with it in order to form a bed during pressing and the material expressed through linen in a warmed meat press. It was found necessary to dismantle the press and remix its contents twice during the process. The extract was cooled to 0° C. and filtered from the separated Na_2SO_4. $10H_2O$; the filtrate (1100 to 1200 ml.) represented a yield of 66 per cent. on the basis of the water content of the yeast, and at a dilution corresponding to 1 g./ml. (that chosen as standard for testing purposes) contained 20·4 mg. dry solids/ml. and 0·23 mg. total N/ml.

An extract prepared by boiling yeast with twice its weight of water and decanting and filtering was equally active on a basis of the quantity of yeast used, but was much more coloured than the Na_2SO_4 extract, and at an equivalent concentration contained 45 mg. dry solids/ml. and 2·8 mg. N/ml.

Lead and mercury precipitation.—The whole of the Na_2SO_4 extract was stirred and lead nitrate added till in excess while progressively neutralizing with NaOH. After keeping for ½ hour the precipitate was filtered off and washed with water. The filtrate and washings were similarly precipitated with mercuric nitrate. The filtrate was freed from lead and mercury with H_2S and concentrated *in vacuo* to 200 ml.

Amyl alcohol extraction.—This solution was acidified with H_2SO_4 and extracted by shaking with amyl alcohol. The amyl alcohol was extracted with a little acidified water and then with excess baryta. The alkaline extracts were neutralized with sulphuric acid and kept in this form, freeing from Ba by Na_2SO_4 before testing. Activity was found only in the extractives, though the activity of these was only about one-fifth of that of the original yeast extract. In dilution equivalent to 1 g. yeast/ml. the Ba-free solution contained 0·3 mg. dry weight/ml. and 0·0075 mg. total N/ml. Recombination of the various fractions showed that destruction of the factor had occurred, and not its separation into portions individually inactive. Simple standing with acid and alkali did not cause loss, which was apparently due to acid alcohol treatment.

ACIDIC NATURE OF THE FACTOR AND ITS ATTEMPTED REPLACEMENT BY KNOWN COMPOUNDS.

The material was also extracted from the acidified mercury filtrate with ether and could be washed from non-aqueous solution by $NaHCO_3$. These properties indicated a carboxylic acid type of compound, which was confirmed

by preparation of a methyl ester by standing the concentrates with methyl alcoholic H_2SO_4. An inactive ester was separated which regained part of its activity on hydrolysis with dilute H_2SO_4.

Lecithin and various fats and oils, before and after mild hydrolyses, did not replace the factor. The properties of the compound which were known at this stage were such that it appeared different from known biochemically important acidic compounds. Sporogenes vitamin (Pappenheimer, 1935) and succinic acid (pyruvic acid dehydrogenase activator; Annau and Erdös, 1939) did not replace it. The properties of pantothenic acid also appeared different, but following the publications of Williams *et al.* (1939) and of Wooley, Waismann and Elvejhem (1939) establishing pantothenic acid as an amide of a dihydroxyvaleric acid and β-alanine, it was thought possible that the dihydroxyvaleric acid might alone appear as a bacterial growth factor, as has the other component, β-alanine (Mueller, 1937). A specimen of calcium pantothenate concentrates from Prof. R. J. Williams was obtained through Dr. W. C. Evans, who also informed us that it had been found necessary for the growth of some strains of *C. diphtheriæ*. This specimen, when present in the medium in a concentration of 0·1 μg./ml. completely replaced the present factor. The effect on growth of 0·004 μg./ml. was evident in 16½ hours. Mild hydrolysis of pantothenic acid, which inactivates it for the growth of yeast (Williams *et al.*, 1933), lactic acid bacteria (Snell, Strong and Peterson, 1937) and *C. diphtheriæ* strains (Evans *et al.*, 1939), inactivated it also for growth of streptococcus.

Though the active material when in yeast extract had differed in properties from those recorded for pantothenic acid, in the barium salt concentrates it behaved exactly as pantothenic acid. They were unaffected by ½ hour at 120° C. or longer heating at 100° C., but completely destroyed by *N* H_2SO_4 at 100° C. At pH 3·6 almost the whole of the active material was adsorbed by norite. A comparison of titrations indicated the barium salts to contain about 2 per cent. of pantothenic acid. The recorded properties of pantothenic acid are entirely consistent with its presence in the barium salt fraction; partial destruction with acid alcohol (p. 331) has also previously been noted by Williams *et al.* (1938). The barium salts were converted into brucine salts by brucine sulphate; these were water-soluble, and had approximately the same solubility in chloroform as those of Williams's pantothenic acid. Application of the method of Snell *et al.* (1937) and of the preliminary part of the method of Williams *et al.* (1938) indicated the former to be superior when working with yeast as starting material, and to yield concentrates of approximately the same activity as the procedure outlined above. Attempts at a more satisfactory process were in progress when the letter of Subbarow and Rane (1939) indicating the necessity of pantothenic acid for the Dochez NY5 strain of hæmolytic streptococcus was published; in view of this, further work has been postponed until pantothenic acid can be synthesized.

DISCUSSION.

Woolley *et al.* (1939) showed that acid hydrolysis of pantothenic acid under the conditions employed above resulted in split of the amide linkage,

leaving substantially intact the dihydroxyvaleric acid. The failure of streptococcus to grow in the present medium in the presence of the products of this hydrolysis shows that the organism has been unable to re-form this amide. This can be compared with the limited ability of streptococcus to utilize glutamic acid in place of glutamine (McIlwain *et al.*, 1939), though the organism obviously forms the amide linkages of its own proteins. The amide linkages in both glutamine and pantothenic acid are however unusual in not being in the α-position to either an amino or a carboxylic acid grouping (in glutamine, γ, and in pantothenic acid, β). The absence of similar vicinal groupings was shown by Bergmann, Zervas and Fruton (1936) to limit the activity of papain peptidase. *Staphylococcus aureus* was also found similar in requiring for growth much higher concentrations of nicotinic acid than of nicotinamide (Knight and McIlwain, 1938), indicating that difficulty was found in the synthesis of this amide.

SUMMARY.

Pantothenic acid concentrates in high dilution caused growth of the "Richards" strain of *Streptococcus hæmolyticus* in an otherwise deficient medium.

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GLUTAMINE AND THE GROWTH OF BACTERIA.

PAUL FILDES AND G. P. GLADSTONE.*

From the Department of Bacterial Chemistry (Medical Research Council), Bland-Sutton Institute of Pathology, and from the Courtauld Institute of Biochemistry, Middlesex Hospital, London.

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IN a recent paper (McIlwain, Fildes, Gladstone and Knight, 1939) it was shown that certain hæmolytic streptococci, when inoculated into a particular medium in numbers approximating to 1 million, were incapable of growth unless glutamine was added also, in a concentration $M/5000$ for maximum effect. It was further shown that growth would take place, though less actively, if glutamate were added instead of glutamine in a concentration 100 times greater.

It was argued that glutamine is essential for the growth of the streptococcus, but that 1 million cocci in the presence of high concentrations of glutamate were able to synthesize glutamine from glutamate sufficiently actively for growth to take place. It was deduced that this synthesis was potentially possible from the fact that the organism readily became altered, and then could grow without glutamine or excess of glutamate.

The present paper records further observations on the action of glutamine. The technique followed that of McIlwain *et al.* except that uracil, cytosine, thymine and guanine were omitted from the medium, and Fe was added as indicated in Experiment 1.

A. FURTHER OBSERVATIONS ON GLUTAMINE AND GROUP A STREPTOCOCCI.

Conditions Affecting Growth Without Glutamine.

The difficulty already referred to in maintaining the inoculum constant so that it would not grow without glutamine led to further observations of this phenomenon. If the assumption was correct that the growth took place through synthesis of glutamine from glutamate, this process should be hindered by reduction in the amount of enzyme, and might be facilitated by increasing the concentration of glutamate. It was also noted that the tendency for growth to take place in the absence of glutamine was increased by the presence of Fe.

In Experiment 1 the effect of variation in the inoculum is shown, together with that of added glutamate. Fe was added in all cases. It is seen that 1 million cocci grew slowly without glutamine and 10,000 cocci still more slowly. The growth in the presence of added glutamate was more rapid. With 1000 cocci no growth occurred in 8 days whether glutamate was added or not, but this reduced number of cocci grew with normal rapidity in the presence of glutamine.

* Leverhulme Research Fellow.

Experiment 1.—Technique as in McIlwain *et al.* (1939), with, however, the addition per tube of 0·25 c.c. $M/500$ ferrous ammonium sulphate in $N/50$ HCl and the omission of the bases. The cocci were raked from an agar slope and made up with the basal buffered peptone to a standard opacity of which 0·1 c.c. represented 1 million cocci. Tenfold dilutions of this were made with the peptone. Glutamate was added to a concentration $M/5000$ additional to that in the peptone. Glutamine in the control was $M/5000$.

	No. of cocci.	Hours and days. 19.	25.	43.	3.	4.	5.	7.	8.
Complete medium + 0	10^6	0	0	?	++	+++	++++	++++	++++
„ „	10^5	0	0	0	0	0	0	++	++
„ „	10^4	0	0	0	0	0	0	tr.	+
„ „	10^3	0	0	0	0	0	0	0	0
„ + glutamate	10^6	0	0	?	++	+++	++++	++++	++++
„ „	10^5	0	0	0	0	++	+++	++++	++++
„ „	10^4	0	0	0	0	+++	++++	++++	++++
„ „	10^3	0	0	0	0	0	0	0	0
„ + glutamine	10^3	++++	++++	++++	++++	++++	++++	++++	++++

Here and elsewhere the + signs are roughly proportional to the mass of growth. tr. = trace.

Experiment 2.—As in Experiment 1, but glutamate $M/100$.

	No. of cocci.	Hours and days. 17.	42.	3.	4.	5.	7.
Complete medium + 0	10^6	0	0	0	0	++++	++++
„ „	10^5	0	0	0	0	0	0
„ „	10^4	0	0	0	0	0	0
„ „	10^3	0	0	0	0	0	0
„ + glutamate	10^6	0	+	++	+++	++++	++++
„ „	10^5	0	0	0	0	+	++++
„ „	10^4	0	0	0	0	0	0
„ „	10^3	0	0	0	0	0	0
„ + glutamine	10^3	++++	++++	++++	++++	++++	++++

In Experiment 1 the cocci had been passaged in a mouse 4 weeks before the test. In Experiment 2 the test was repeated with cocci which had been recovered directly from the mouse. In this case growth took place from 1 million cocci only on the 5th day and not with 100,000 in 7 days. With added glutamate growth was more rapid and took place with 100,000. This result illustrates the fact that a recently passaged strain is more dependent upon added glutamine than a strain which has been passaged less recently.

Since 1000 cocci grew normally in these experiments in the presence of glutamine, but did not grow without glutamine even when glutamate was added, this number of cocci was used as a standard in further experiments.

The Effective Concentration of Glutamine.

The experiments of McIlwain *et al.* were usually confined to short periods of observation, and the impression was gained that maximum growth required a relatively high concentration of glutamine. Quantitative experiments have been repeated, using 1000 cocci as inoculum from a recently passaged culture. Experiment 3 shows the effect of falling quantities of glutamine tested with added glutamate and without. It will be seen that glutamine alone causes growth in a concentration as little as $M/2{\cdot}5$ million, and that the mass of growth is maximal whenever growth takes place at all. The effect of glutamate is to accelerate growth, but not to cause growth with an even smaller concentration of glutamine. The amount of glutamine necessary to show an effect is of the same order as is necessary with other vitamin-like growth factors (about $0{\cdot}5\gamma$ per tube).

Variation in the Glutamine Requirements of the Strain " Richards ".

It was noted by McIlwain *et al.* (1939) that after a number of subcultures on agar the test strain " Richards " became able to grow in the absence of added glutamine, and this was ascribed to a development of a power to synthesize glutamine induced by a deficiency of this essential in the agar. This development of a power to grow without an essential growth substance induced by alteration in the nutrient environment is the phenomenon of " training " familiar in this type of work. The technique consists in depriving the bacterium of a growth substance, and offering in place another material from which it is thought that the growth substance can be synthesized.

Experiment 4 illustrates the process in the present case. The original culture was able to grow only from an inoculum of 1 million cocci, and on the 5th day. In the presence of glutamate, however, it grew from 100,000 cocci in 6 days. In the first subculture on glutamate a " chance " variation took place in the two tubes containing few cocci which produced individuals which could grow without glutamine. These, on further subculture, maintained this characteristic.

Experiment 4.—The technique was as in Experiment 1. The medium and the medium + added glutamate ($M/100$ final) were inoculated with dilutions of the streptococci representing 1 million to 1000 cocci per tube. The figures indicate the number of days before growth was observed. Serial subcultures,

Experiment 3.—As in Experiment 1. Glutamate added to *M*/100.

	Molarity of glutamine.		Hours or days after inoculation.					
			20.	42.	3.	4.	5.	7.
Complete medium + 0	.	.	0	0	0	0	0	0
,, + glutamine	. $2{\cdot}5 \times 10^{-4}$	.	++++	++++	++++	++++	++++	++++
,, ,,	. 5×10^{-5}	.	+	++	++++	++++	++++	++++
,, ,,	. 10^{-5}	.	0	tr.	++++	++++	++++	++++
,, ,,	. 2×10^{-6}	.	0	0	tr.	+	++++	++++
,, ,,	. 4×10^{-7}	.	0	0	0	tr.	++++	++++
,, ,,	. 8×10^{-8}	.	0	0	0	0	0	0
,, ,,	. $2{\cdot}5 \times 10^{-4}$ + glutamate	.	++++	++++	++++	++++	++++	++++
,, ,,	. 5×10^{-5} ,,	.	+++	++++	++++	++++	++++	++++
,, ,,	. 10^{-5} ,,	.	tr.	++++	++++	++++	++++	++++
,, ,,	. 2×10^{-6} ,,	.	0	++++	++++	++++	++++	++++
,, ,,	. 4×10^{-7} ,,	.	0	0	++++	++++	++++	++++
,, ,,	. 8×10^{-8} ,,	.	0	0	0	0	0	0
Complete medium + glutamate	. .	.	0	0	0	0	0	0

also serially diluted, were made from tubes marked *. 0 = no growth in 8 days. A positive culture, $M/5000$ glutamine, permitted growth from 1000 cocci overnight.

	Day on which growth was observed from different numbers of cocci.			
	10^6.	10^5.	10^4.	10^3.
Medium alone	5	0	0	0
Medium + glutamate	2*	6	0	0
,, ,, . 1st subculture	4	5	3	2*
,, ,, . 2nd ,,	2	3	3	4*
,, ,, . 3rd ,,	1	1	2	3*
,, ,, . 4th ,,	1	1	2	2*

Discussion.

It would appear that a possible explanation of the phenomena observed in these experiments might be sought along the lines indicated in previous papers, namely that the necessity for the addition of a growth substance is due to a failure of the organism to synthesize it, and the dispensability of a growth substance is due to its synthesis by the organism.

According to this line of argument we should look upon the strain "Richards" as ordinarily met with in its pathogenic state as an organism which uses glutamine in the metabolic processes of growth, but that it has become modified by the evolutionary influences discussed in particular by Lwoff (1932) and Knight (1936). This modification consists of a loss of functioning enzymes, which it formerly possessed, capable of synthesizing glutamine from other ingredients of the medium. This loss cannot be absolute, since when the number of cocci is large enough, glutamine appears to be synthesized, especially when glutamate is in high concentration and Fe is present. When the enzymes are thus brought again into activity, they increase in concentration per cell on subculture in the substrate, so that subsequently an equal synthetic power is exerted by a smaller number of cells.

The experimental evidence on which this argument is based is admittedly slight. It consists of a demonstration by Fildes, Gladstone and Knight (1933) and Fildes and Knight (1933) that bacteria which cannot grow unless supplied with tryptophane can nevertheless be "trained" to grow without tryptophane, and can then be proved to synthesize it. In the case of the streptococcus and glutamine, however, we have not yet been able to demonstrate that glutamine is, in fact, synthesized. Experiments were designed to test this point with suspensions of cocci along the lines used by Krebs (1935), who found a synthesis of glutamine from glutamate by the action of certain tissues, but these were unsuccessful. Negative results with suspensions are not, however, important in the absence of any knowledge of the correct conditions.

Experiment 3 shows an effect of glutamine which is different from those observed with other growth factors. In the case of nicotinic acid, for instance, the concentration of this substance in the sub-optimal range is proportional to the mass of growth (Fildes, 1938*a*), a certain number of molecules, as cozymase, being presumed to be present in each cell. With glutamine in the case of streptococcus this is not, however, the case. When growth takes place at all, it becomes maximal. This observation appears to be in favour of the view

that when growth is started with a minimal quantity of glutamine, more glutamine can be synthesized from some constituent of the medium.

This synthesis, however, does not depend upon an alteration in the powers of the cocci of the type illustrated in Experiment 4. The growth which developed in Experiment 3 from 4×10^{-7} *M* glutamine was on the 7th day diluted and 1000 cocci inoculated into the same medium without glutamine and with *M*/5000 glutamine. With glutamine growth occurred normally overnight ; without glutamine no growth appeared in 7 days. It would therefore appear that the normal untrained streptococcus can only start the process of growth in the presence of glutamine, but that when growth has started it proceeds in the same manner as it would in the presence of excess glutamine.

B. GLUTAMINE AND OTHER STRAINS OF STREPTOCOCCI AND OTHER BACTERIA.

McIlwain *et al.* (1939) showed that the action of glutamine was manifested by 11 strains of hæmolytic streptococci, but that 2 others grew without this nutrient. These strains were not, however, subdivided into their groups.

The following are the results of tests on various bacteria, in all cases the inoculum being about 1000 organisms :

Hæmolytic. *Streptococci.*

(a) *Group A.*—Nineteen strains were tested in the Bacto-peptone medium with and without *M* 5000 glutamine. No Fe was added. In all cases the cocci grew overnight with glutamine but did not grow in 4 days without. Of the 19 strains, 11 were isolated from the throat or nose.

(b) *Group B.*—Ten strains were tested under the same conditions, and of these 8 grew equally rapidly without glutamine as with, while 2 grew some 20 hours sooner with glutamine. All strains were of veterinary origin.

(c) *Group C.*—Of 3 strains from the throat, one did not grow without glutamine.

(d) *Group E.*—One strain required glutamine.

(e) *Group F.*—One strain required glutamine.

(f) *Group G.*—Of two strains, one required glutamine and the other grew more quickly with glutamine.

Viridans.

Four strains all required glutamine.

Fæcalis.

Three strains grew equally well without glutamine.

It is thus seen that glutamine has an influence on the growth of all varieties of hæmolytic and viridans streptococci tested. There is, however, a striking difference between the behaviour of Groups A and B. Whereas no Group A strain grew without glutamine, all Group B eventually grew under these conditions.

Pneumococcus.

One strain (Type 1) has been tested, the inoculum being about 1000 cocci from a broth culture containing 1 per cent. horse-serum. This strain would grow only sporadically on the streptococcus medium under aërobic conditions, but anaërobic tests gave constant results. In the presence of $M/5000$ glutamine full growth occurred overnight, but without glutamine in 2 cases growth was delayed till the 3rd day, and in one was not observed during 5 days. It is thus clear that glutamine has an important function in the growth of pneumococcus. The requirement for glutamine as a nutrient is probably not so absolute as with Group A streptococci, since this strain of pneumococcus grew readily without glutamine in the presence of a high concentration of glutamate.

Other Bacteria.

It is already known that several species of bacteria grow on mixtures which do not contain glutamine, but since growth in these mixtures is sometimes slow, it was possible that glutamine might exert an accelerating effect, as in the case of the two Group B streptococci already mentioned.

The conditions under which the tests were carried out varied with the different species.

B. anthracis.

Six strains were examined according to the growth technique of Gladstone (1939). The growths of five were accelerated by the addition of $M/1000$ glutamine. The remaining strain grew equally without glutamine.

Proteus.

One strain was tested by the method of Fildes (1938*a*). There was a marked stimulation of growth by glutamine. Glutamic acid produced almost as great a stimulation. Asparagine had no effect, showing that the effect was not due to amino-nitrogen added to a basal ammonia medium.

Bact. coli.

One strain when grown under the conditions described by Fildes (1938*b*) showed no acceleration of growth when a 24-hour culture was used as inoculum, but a constant acceleration when a culture was used after keeping for 14 days on the bench.

H. influenzæ.

One strain was tested under conditions described by Lwoff and Lwoff (1937), using, however, Bacto-peptone and hæmatin. The addition of glutamine led to a trivial acceleration.

C. diphtheriæ.

Six strains of each of the *gravis*, *mitis* and *intermediate* varieties, recently isolated, tested under the conditions used for streptococcus, appeared to be indifferent to the addition of glutamine, although two of the *intermediates* when first tested showed a slight acceleration.

Staphylococcus.

One strain tested under the conditions described by Gladstone (1937) showed some acceleration in growth on the addition of glutamine.

Gonococcus.

It was found that the gonococcus would grow on the mixture used by McIlwain *et al.* if the procedure of Glass and Kennett (1939) was adopted. To each tube 1·0 c.c. of an autoclaved 10 per cent. suspension of blood charcoal was added. Growth was equal whether glutamine was added or not.

Discussion.

In the light of the experiments in Section A of this paper it would appear that glutamine is associated with the growth of all pathogenic streptococci, but that owing to the differences in synthetic power of different strains induced by differences in their previous environments, the indispensability or otherwise of this substance as a nutrient for particular samples is variable. The fact that glutamine has also a nutrient effect, however slight, on other bacteria suggests that it is associated with the growth metabolism of these bacteria also, and that it has, in fact, a general bearing upon bacterial growth.

CONCLUSIONS.

The indispensability of glutamine as a growth factor is most marked in the case of Group A hæmolytic streptococci and least in Group B. The view that the indispensability or otherwise depends upon the glutamine-synthesizing powers of different strains is advanced but not proved. There is reason to suppose that glutamine is associated with the metabolism of other bacteria.

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THE EXTRAVASCULAR DEVELOPMENT OF THE MONOCYTE OBSERVED *IN VIVO*.

R. H. EBERT AND H. W. FLOREY.

From the Sir William Dunn School of Pathology, University of Oxford.

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TECHNIQUE.

In the following paper a description is given of the extravascular development of the monocyte in living growing tissue, the observations being made with modified Sandison-Clark chambers prepared in rabbits by a method previously reported (Ebert, Florey and Pullinger, 1939). Further experience with these chambers has enabled us to introduce certain modifications for increased ease of observation.

(1) The previously described chambers had transparent tissue approximately 50μ thick, but we have found it possible to produce regularly areas for observation 30μ thick by reducing the thickness of the buffers. Organization of the clot on the central table of the chamber takes longer in the thinner chambers, and no lymphatics have been observed to grow into them, but the possibilities of watching and photographing individual cells are greatly improved. Some trouble has also been experienced in preventing growth over the central removable plug even when this is apparently pushed into contact with the mica coverslip. This is due to the difficulty of keeping the space between the plug and the coverslip entirely free from blood-clot. This occurrence, however, was found to have a great advantage in producing a very thin sheet of vascularized tissue. The vessels in the tissue 30μ thick on the table are capable of growing into this thinner space, so that areas 1 or 2 mm. in diameter (according to the size of the central plug) of vascular tissue not more than 15μ thick have been produced. Vessels grow only with difficulty, if at all, from the thick surrounding tissue on to the central table into a space as thin as 15μ. To prevent vessels from growing over the central plug area it was found necessary either to be certain no debris existed on the plug or to give the plug a twist every day.

(2) *Removal of vascular area over the central plug.*—It was found useful for observing the reaction of cells to injury in organized tissue to cut away the portion over the central plug. This was done as follows: the central plug was pulled according to the method previously described. A sterile steel punch, slightly less in diameter than the plug-hole, was then gently inserted and rotated. This punch was very sharp, and rotation with only slight pressure removed a circular portion of the vascularized tissue. The punch was then withdrawn and the hole flushed out with sterile saline to remove debris. While these punching operations were being carried out the extracted plug was cleaned, immersed in 10 per cent. formalin for 10 minutes and then washed

in sterile saline. The plug was then replaced. In no case was infection introduced. This procedure had, however, one disadvantage, in that the area next to the cut edge, in which observations were particularly necessary, was optically the most unsatisfactory owing to the flange on the plug. To obviate this the following procedure was devised. A chamber with a 3-mm. central plug was made and inserted in a rabbit's ear. After the table was vascularized and stabilized, including the area over the plug, the plug was removed and replaced by a 3-mm. plug with a 1-mm. hole through its centre. The sharp punch was then used to cut out a 1-mm. diameter portion of tissue as before described. The plug with the 1-mm. hole was then removed and the original solid plug replaced. By this means the cut edge was over a completely transparent portion of the central plug.

(3) An improvement was made in the method of fixing the chamber and ear to the mechanical stage of the microscope by employing the device illustrated in Fig. 1. Its advantages are that it holds the chamber rigidly during observations, and makes unnecessary the use of nembutal anæsthesia even for the most exacting photographic work. It also holds the chamber so that the coverslip is always parallel to the surface of the objective lens. Improvements in the substage condenser system have also been made to give better illumination. The two top lenses of a Watson holoscopic condenser have been removed and the remaining two lenses mounted on a brass extension collar, so that light from a Zeiss or Watson microscope lamp can be focused on the transparent tissue of the chamber, which is very considerably above the level of the ordinary microscope stage. With this improved substage lighting it became of advantage to use a Leitz 2 mm. apochromatic lens and periplanatic or compensating eye-pieces for critical observation and photography.*

After trial of many types of film, Kodak E.F. pan. has been found to give the best results. Additional contrast with red objects has been obtained by using a Wratten B filter.

Vital staining.—As a preliminary to a microscopic study *in vivo* of tissue reactions to the tubercle bacillus and its chemical fractions, it became necessary to identify and follow the behaviour of the macrophages, which occur at and in advance of the edge of the capillaries growing into the space over the central table (Fig. 3). For this purpose vital staining was employed. Since the work of Bouffard (1906) and Goldmann (1909) on intravitam staining, the use of vital dyes has been the accepted method for studying the distribution and function of certain fixed tissue elements. It is unnecessary here to discuss the question of how the dye enters the cell, or whether the granules represent preformed structures or are elements merely formed in reaction to the dye. These problems have been adequately dealt with elsewhere (Evans and Scott, 1920–21 ; Cappell, 1929*a*, 1929*b* ; Jaffé, 1938).

It is a well-recognized observation that certain acid diazo dyes are readily taken up in granular form by certain tissue-cells, and that they are not as a rule taken up by blood-cells (Cappell, 1929*a*). Three dyes, which were given intravenously, have been used for the present work—vital new red (Cappell, 1929*a*), pontamine sky blue (Hudack and McMaster, 1932) and trypan blue.

Vital new red was used for most observations because the red granules

* For these two improvements we are indebted to Dr. A. G. Sanders.

gave better photographic results. Of this dye 10 c.c. of a 3·5 per cent. solution were given intravenously every 2–3 days to rabbits weighting 2·5–3 kg. After four or five injections certain tissue-cells were usually brilliantly stained. Vital new red staining fades very slowly. Pontamine sky blue was used in 5 per cent. solution in distilled water—a 5 c.c. dose being given every 2–3 days. After 4–5 injections certain tissue-cells in the chamber were well stained. Pontamine blue fades more rapidly than vital new red and trypan blue. Trypan blue (10 c.c. of 1 per cent. solution) given every 2–3 days has also been used. After 3–4 doses the cells are deeply stained.

Supra vital staining.—Dry films of neutral red (Grübler's) prepared according to Cappell's directions (1929*a*) were used to study supravital preparations of rabbit blood.

OBSERVATIONS.

By the combination of vital dyes with visual and photographic observation it has been possible to follow the extravascular development of the monocyte in some detail. The results obtained will be first of all briefly summarized, to be followed by specific illustrations of the various stages of development noted.

The monocyte migrates from a blood-vessel into an organizing clot or in response to a mechanical injury. Once in the tissue it undergoes considerable increase in size from about 10μ (in the rounded state) up to an average of 30μ. The size of these large cells, which will here be called macrophages, varies considerably, and giant-cells developed from them may be met with up to 130–140μ in diameter (Fig. 8). These macrophages ingest and digest the blood-clot. They also take up any debris present such as particles of mica or globules of vaseline. They appear at the growing edge of the capillaries and slightly in advance of them (Figs. 3, 4). After vital new red injections they appear to be diffusely stained bright pink or red, and but few distinct dye-granules can be made out, though they contain particles of debris, pigment and certain refractile droplets. They are for the most part rounded in spite of their evident activity in dealing with the clot. This may partly be due to the fact that it is fairly certain that this very thin tissue in the ear is colder than that of the body generally, so that it is probable that all the healing processes are slowed down. The macrophages present at any time do not tend to advance with the growing capillary edge, but this region is frequently so fluid that probably they are carried there passively. Usually they remain near the position in which they first appear, and undergo further development while the growing capillaries advance beyond them. This further development consists in a certain clarification of the cell protoplasm during which colourless refractile droplets appear, principally at the periphery of the cell, while the dye is concentrated more centrally. The refractile droplets gradually disappear and deep red staining granules make their appearance (Fig. 6). At the same time a clear space, corresponding to the nucleus, appears towards one side of the cell. During this period of development the cells tend to orient themselves along the blood-vessels and become elongated in the axis parallel to the vessels (Figs. 5, 6), though many are distributed in the connective tissue.

At this stage these cells appear to have all the characteristics attributed to histiocytes. They remain relatively immobile, changing their shape but never moving very far in quiescent tissue (Figs. 33–36). With increasing age the fine regular granules of dyed material tend to coalesce to form larger granules, while at the same time the cells become smaller, though retaining their position close to vessels (Fig. 7). This series of changes is illustrated diagrammatically in Fig. 2. Increase in the size of the granules may be related to the amount of dye given (Evans and Scott, 1920–21), but it often occurs in rabbits long after the injections have been stopped. Crystals of dye are also seen in older cells.

It is quite possible that macrophages at the growing edge divide, although this has never actually been observed. A monocyte which migrated into an injured region and changed into a macrophage was seen to divide, however (Fig. 30), and division of individual histiocytes has been seen.

It is to be emphasized that this series of changes is not inferential in the sense that successive phases in different cells have been correlated to make a plausible succession, but are the result of following individual cells through the cycle. It is relatively simple to follow the stage from the first formed macrophage to the histiocyte, and this was done with seven different cells or groups of cells (Figs. 9–16 ; Fig. 55). More difficulty was found in making certain that the macrophages at the growing tissue edge were, in fact, monocytes which had migrated from the blood-stream. This might at first appear to be simple, but it must be remembered that it is impossible to keep an animal under continuous observation for days on end. Advantage was taken of the fact that after intravenous injection of indian ink the only blood-cells to take up significant quantities of carbon are the monocytes (Fig. 24). Sometimes polymorphs take up some particles, but fortunately these are easily recognized in the tissue of the chamber after they have migrated. Gurr's indian ink (5 c.c. of 20 per cent. solution) was injected intravenously. Observations were made on the growing edge of an injured region and ink-containing monocytes observed to emigrate into the organizing clot. One of these cells was chosen and observations made every 2 to 3 hours for the first 36 hours, after which observations were discontinued at night but continued at 3- to 4-hourly intervals during the day. The changes so followed are illustrated in Figs. 17–23 and 24–32. Details relative to these observations will be found in the descriptions attached to the figures.

Though it was thus made certain that the blood monocyte was capable of developing extravascularly into a cell indistinguishable from that frequently called a histiocyte, it was not quite clear that *all* the macrophages present at the developing edge originated from monocytes. It is conceivable that some were due to the mobilization and division of pre-existing tissue histiocytes. Two forms of observation make this supposition unlikely.

(1) *The reaction to mechanical injury.*—By the technique previously described it was possible to remove a circular piece of vascularized tissue from the centre of the table. Blood-clot accumulated in the area from which the tissue was removed. This was then vascularized by vessels growing in from the tissue on the table. This undisturbed tissue had in it large numbers of histiocytes stained red. Stained cells near the cut edge were selected for

observation. These cells, though changing their shape and position very slightly, did not migrate into the newly organizing tissue, though large numbers of unstained macrophages made their appearance. These macrophages remained unstained provided no injections of dye were given during the week before the cutting operation. This observation, which was repeated several times, makes it very improbable that the macrophages appearing in the organizing tissue originated from histiocytes previously present in the surrounding normal tissue (Figs. 46–51).

(2) *Double staining.*—A central piece of tissue was cut out from a chamber in an animal whose histiocytes were brilliantly stained with vital new red. The animal was then given injections of trypan blue. The macrophages and subsequent histiocytes appearing in the newly vascularized area where the cut had been made were stained blue, but the histiocytes in the older tissue remained red or took on a purple tinge. Occasionally one or two histiocytes containing red dye-granules were seen in the freshly organized tissue on the central pin, but this was quite exceptional. Even if these cells were derived from old histiocytes they were probably passively carried into the injured region (which is often fluid), since histiocytes at the edge of the injured region which were carefully followed for days were not seen to migrate more than a few μ. There would thus seem to be little or no connection between the old histiocytes produced in the first vascularization of the table and the new-formed cells when the small central cut-out area was vascularized.

From observations in thick chambers into which lymphatics have grown it is clear that a certain number of macrophages enter the lymphatics, as already described by Clark and his collaborators (Clark, Clark and Rex, 1936). There is, however, a very sluggish stream in these lymphatic vessels, and it is improbable that any large numbers of macrophages are removed from the newly vascularized tissue in this way.

Functional capacity of histiocytes.

The cells originating from monocytes which eventually became cells which we are calling histiocytes are indistinguishable from the so-called resting wandering tissue-cells (Maximow, 1902). As our observations show, the capacity for wandering, at least under the present experimental conditions, is very limited. They certainly change their shape continuously but do not appear to progress far in the tissues, although a demand for macrophages has been created in their immediate neighbourhood (Figs. 46–51). Nevertheless, they fully preserve their original phagocytic and digestive powers. These properties were strikingly illustrated by causing small hæmorrhages in circumscribed areas by pressing on the mica coverslip. This was most conveniently done by mounting a glass rod with a fine rounded tip in a Leitz micro-dissector. The tip of this rod was manipulated into position under a 16-mm. objective and then pressure exerted in a selected place. Another and less satisfactory method was to press an objective into contact with the coverslip. Small hæmorrhages were caused in this way along and in the neighbourhood of the blood-vessels. Where histiocytes were present the red cells were cleared away with considerable speed. They were ingested and digested

by the histiocytes (Figs. 37–39 and 40–45). The actual ingestion of red cells has been observed, and the time taken from ingestion to disappearance of the ingested cell was found to be about 2 hours. It was noted that the small hæmorrhages were cleared away entirely by the histiocytes—there was no fresh migration of monocytes. It was also observed that red cells outside the immediate range of the histiocyte remained for considerable periods free in the tissues without causing migration towards them of either histiocytes or newly-emigrated monocytes.

Thus, although the mature histiocyte retains perfectly the phagocytic powers of the freshly-migrated monocyte, it does not revert when ingesting material to the rounded form described as existing in the growing edge of healing tissue, nor do the unstained refractile droplets reappear.

With increasing age the histiocytes become reduced in size (Fig. 6), and there is evidence either of phagocytosis of one cell by another or fusion (Figs. 43, 44).

Reactions to vaseline.

It has been the practice to seal off the central removable plug with vaseline. In some cases, with a relatively loosely-fitting plug, some vaseline has seeped up into the tissue on the table. It has been possible to observe the reaction of both the macrophage and histiocyte (Figs. 53, 54) to this substance. Both types phagocytose the material, the histiocyte maintaining its position and general appearance. An interesting result was obtained in a chamber in which some vaseline was accidentally introduced during the removal and replacement of the central plug. This vaseline was ingested in large quantities by numerous macrophages and, in the course of the organization of this region, these accumulated at the central portion of the plug area. Here they have remained for 11 months without apparently undergoing the usual change into the histiocyte type of cell (Fig. 52). Although this animal has been given many injections of vital new red it was not possible to stain these macrophages at all, though the histiocytes outside this area became brilliantly stained.

Reaction to direct injury.

Vitally stained histiocytes which have been injured appear to fade. After the tissue over the central plug has been cut away directly, injured histiocytes along the cut edge gradually fade until they can no longer be seen. The same phenomenon can be observed in a cauterized region, only in this case the fading is more rapid. Histiocytes in the burned area immediately stain diffusely, and after several hours completely lose their dye.

Further observations.

In addition to the histiocyte which comes, in many cases, to lie alongside blood-vessels, other adventitial cells are found. These lie in close apposition to the walls of the finer blood-vessels and do not take up any vital stain. Fig. 57 illustrates histiocytes, an adventitial cell and an endothelial nucleus for comparison. The origin of these adventitial cells has not been determined and their function is the subject of further investigation.

In some preparations after the replacement of a chamber as previously

described (Ebert, Florey and Pullinger, 1939), a space of 1 mm. or more is left between the tissue of the ear and one edge of the table. Though this space may be filled with blood-clot to begin with it is not always vascularized, but in the course of time becomes filled with colourless fluid in which are cells of different types. Some of these become attached to the mica coverslip. They present very varied shapes and are of widely varying sizes, but all are characterized by large colourless refractile droplets and a central area, which appears rather diffusely stained red by vital new red (Fig. 56). These cells appear to be identical with epithelioid cells in supravital preparations of tuberculous lesions from the lung of the rabbit (Lewis and Lewis, 1926). Though they probably arise from blood monocytes this has not been definitely proved.

EXPLANATION OF FIGURES.

Figs. 1–8.

Fig. 1.—A bakelite dome glued to the mechanical stage of the microscope. There is a loose chamber on top of the dome to show how this is held. (× 1·5).

Fig. 2.—The series of changes from a monocyte to a histiocyte shown diagrammatically. A, monocyte; B, macrophage; B_1, giant-cell; C, D, transition forms between a macrophage and a histiocyte; E, a young histiocyte; F, an old histiocyte.

Fig. 3.—A growing edge (× 75). The capillary loops are growing into a blood-clot. The macrophages along the growing edge are stained with vital new red.

Fig. 4.—Typical macrophages (× 515). Note the refractile granules and appearance of diffuse staining: compare the size of the macrophages with that of the leucocytes in vessels.

Fig. 5.—Histiocytes in recently organized tissue (× 105). They are particularly numerous alongside vessels.

Fig. 6.—Young histiocytes in new tissue (× 420). Note the discrete dye-granules and the clear nuclear regions.

Fig. 7.—Old histiocytes lying next to a vessel (× 420). The cells are smaller and the dye is clumped.

Fig. 8.—Giant-cell (× 610).

Figs. 9–16.

This series shows the transformation of three macrophages into typical histiocytes (× 415). Cell (1) was observed in front of a capillary loop 36 hours earlier.

Fig. 9.—Cells (1) and (2) are deeply stained with vital new red. Staining appears diffuse. Refractile granules are concentrated at the periphery of each cell. A clear nuclear region is beginning to appear in cell (2). Cell (3) is faintly stained and refractile granules are evenly distributed throughout the cell.

Fig. 10.—Dec. 13th, 10.30 a.m. (23½ hours). Cell (1) has lost many of its refractile granules and has moved slightly relative to cell (2). Cell (3) has taken up more dye.

Fig. 11.—Dec. 15th, 10.30 a.m. (71½ hours). Cell (1) has moved to the left of cell (2), and both cells have lost their refractile granules. Discrete dye-granules can now be seen in these two cells. The nuclear region has appeared at the edge of cell (1). Cell (3) still contains refractile granules but a clear nuclear region is now visible.

Fig. 12.—Dec. 16th, 10.00 a.m. (95 hours). Discrete dye-granules are more apparent in cells (1) and (2). Cell (3) has lost its refractile granules and dye is beginning to appear in the form of discrete granules.

A fourth cell (cell (4)) has migrated into the field. This cell was present before, and has undergone the same series of changes mentioned above.

Fig. 13.—Dec. 18th, 10.30 a.m. (6 days). All the cells look like typical histiocytes. A fifth cell (5) has migrated into the field and lies between cell (3) and cell (4). It is faintly stained.

Fig. 14.—Dec. 19th, 10.30 a.m. (7 days). The histiocytes have changed their shape and have moved slightly. The surrounding connective tissue is now quite clear.

Fig. 15.—Dec. 20th, 7.00 p.m. (8½ days). The coverslip was accidentally pressed down earlier in the day and the slight injury caused a migration of polymorphonuclear leucocytes. The histiocytes are more clumped but otherwise there has been little change.

Fig. 16.—Dec. 21st, 10.15 a.m. (9 days). Most of the polymorphonuclears have disappeared. The histiocytes are less clumped together.

Figs. 17–23.

Development of monocyte to histiocyte (× 1050).

Fig. 17.—Jan. 26th, 11.45 p.m. A monocyte containing four granules of ink. It is in the unorganized clot several hundred μ in front of the advancing vessels. The faint cell outlines could be seen visually but do not show in the photograph.

Fig. 18.—Jan. 28th, 11.00 a.m. (35½ hours). The monocyte is elongated and the ink-granules are distributed at the two poles of the cell. It has not moved and is closer to the growing-vessels.

Fig. 19.—Jan. 29th, 11.30 a.m. (60 hours). The marked monocyte has increased in size and contains numerous refractile granules. It looks like a typical macrophage. (A series of injections of vital new red was begun only this morning so the cell does not contain any dye.) The marked cell has moved very little, but vessels have grown into the clot and the cell now lies below a capillary loop.

Fig. 20.—Jan. 30th, 4.15 p.m. (89 hours). Vessels have grown well beyond the marked cell which has migrated out from beneath vessel. The clear region of the nucleus has appeared at the edge of the cell, and it has taken up a little vital new red.

Fig. 21.—Jan. 31st (4½ days). The clear nuclear area is at the centre of the cell, and the refractile granules at the periphery are beginning to disappear. A few discrete dye-granules are present. The vessel on the right of the marked cell has contracted since yesterday.

During the next four days the marked cell lost all its refractile granules and took up more dye in granular form.

Fig. 22.—Feb. 5th (8½ days). The marked cell has not moved, but now has a well-stained segregation apparatus. It looks like a typical histiocyte.

Fig. 23.—Feb. 7th (10½ days). The marked cell is more deeply stained, and it is difficult to distinguish between the ink-granules and deeply stained dye-granules in the black-and-white photograph.

In the various photographs the ink-granules appear to vary slightly in size and shape. This is because they constantly change position within the cell and are frequently in slightly different planes.

Figs. 24–32.

Development of monocyte to histiocyte. This change occurred in an injured region.

Fig. 24.—Feb. 14th, 11.00 a.m. A vessel at the edge of an injured region (× 345). Several monocytes containing ink-granules are sticking to the endothelium in an injured vessel and several have migrated into the injured tissue.

Fig. 25.—Feb. 14th, 11.30 p.m. (12½ hours). A monocyte containing a large number of ink-granules inside a vessel which is in stasis (× 485). The changing of this cell into a histiocyte was closely followed.

Fig. 26.—Feb. 16th, 1.30 a.m. (26 hours). Some time between 10.30 p.m., Feb. 15th, and 1.30 a.m., Feb. 16th, the marked cell migrated out of the vessel. It now lies just outside the vessel and the ink-granules are more clumped.

Fig. 27.—Feb. 17th, 12.05 a.m. (52 hours). The marked cell is now about 100μ away from the vessel. Many refractile granules are present, and the clear nuclear region can be seen at the edge of the cell.

Fig. 28.—Feb. 17th, 9.30 p.m. Possibly more refractile granules are present.

Fig. 29.—Feb. 18th, 10.30 a.m. There is a clear nuclear area at the centre of the cell. Some refractile granules have disappeared.

Fig. 30.—Feb. 18th, 3.00 p.m. The marked cell has divided, but the daughter-cells have not separated.

Fig. 31.—Feb. 19th, 11.00 p.m. (5 days). The daughter-cells appear to have separated, but are still very close together. There are fewer refractile granules.

The two marked cells soon lost the remaining refractile granules, and slowly stored vital new red. They soon became indistinguishable from typical histiocytes. The cells never stored very much dye, probably because they did not lie close enough to a blood-vessel. One of the daughter-cells divided again on Feb. 23rd.

Fig. 32.—April 6th. The three daughter-cells are still in the same position, and are typical histiocytes. These cells are still under observation (still present on June 22nd).

Figs. 33–36.—(× 810.)

Fig. 33.—Dec. 18th. This is the histiocyte which developed from macrophage (1) (Figs. 8–15). It contains two large carbon particles.

Fig. 34.—Jan. 26th. The histiocyte has migrated about 100μ in the course of a month. The dye-granules have faded.

Fig. 35.—Jan. 29th. An injection of vital new red was given on Jan. 26th, and the cell has taken up more dye. The shape of the cell and the distribution of ink-particles have changed.

Fig. 36.—Feb. 3rd. Again the shape of the cell and the distribution of dye-granules and ink-particles have changed, but the cell is in the same relative position in the connective tissue. This cell was present a week later but could not be found on Feb. 20th.

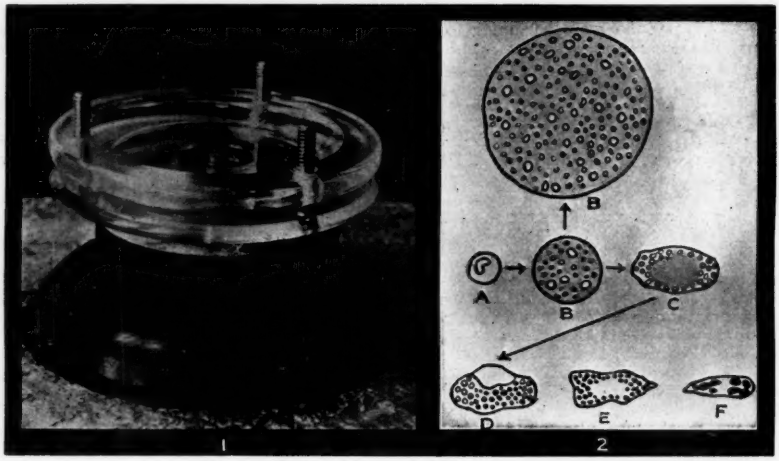
Figs. 37–39.

Reaction to a small local hæmorrhage (× 390).

Fig. 37.—March 13th, 5.45 p.m., 3 hours after injury. Histiocyte (1) contains a droplet of vaseline and is practically hidden by extravasated red cells. The vessels are dilated.

Fig. 38.—March 14th, 10.30 a.m., 20 hours after injury. A large part of the hæmorrhage has been cleared away. Histiocyte (1) has been most active and is packed with ingested red cells. Histiocyte (3) also contains several red cells in various stages of digestion. Histiocyte (2) appears to contain only one large dye vacuole. Histiocyte (4) has moved closer to cells (5) and (6).

Fig. 39.—March 16th, 10.30 a.m., 68 hours after injury. The tissue is practically normal again. A new cell histiocyte (7) has moved into the field and now lies next to histiocyte (1). It did not move until most of the hæmorrhage had been cleared away, however, and took no active part in phagocytosing the extravasated red cells.



Figs. 40–45.

Reaction to small hæmorrhage (× 485).

Fig. 40.—March 13th, 5.35 p.m., 2¾ hours after injury. The hæmorrhage occurred opposite a capillary sprout and for the most part is opposite histiocyte (1).

Fig. 41.—March 13th, 10.35 p.m., 7¾ hours after injury. Histiocyte (1) has already ingested some of the red cells in the hæmorrhage.

Fig. 42.—March 14th, 10.30 a.m., 20 hours after injury. The tissue is almost normal again. Histiocyte (1) contains the remains of several partially digested cells.

Fig. 43.—March 15th, 10.15 a.m., 44 hours after injury. The tissue is entirely normal. Note the three histiocytes below the capillary sprout.

Fig. 44.—March 16th, 10.45 a.m., 68 hours after injury. There are only two histiocytes below the capillary sprout. Histiocyte (3) has disappeared, and histiocyte (2) contains a large dye-vacuole which was not present yesterday. Cell (3) may have been ingested by cell (2) or the two cells may have coalesced.

Fig. 45.—March 17th, 3·00 p.m., 96 hours after injury. The capillary sprout has retracted.

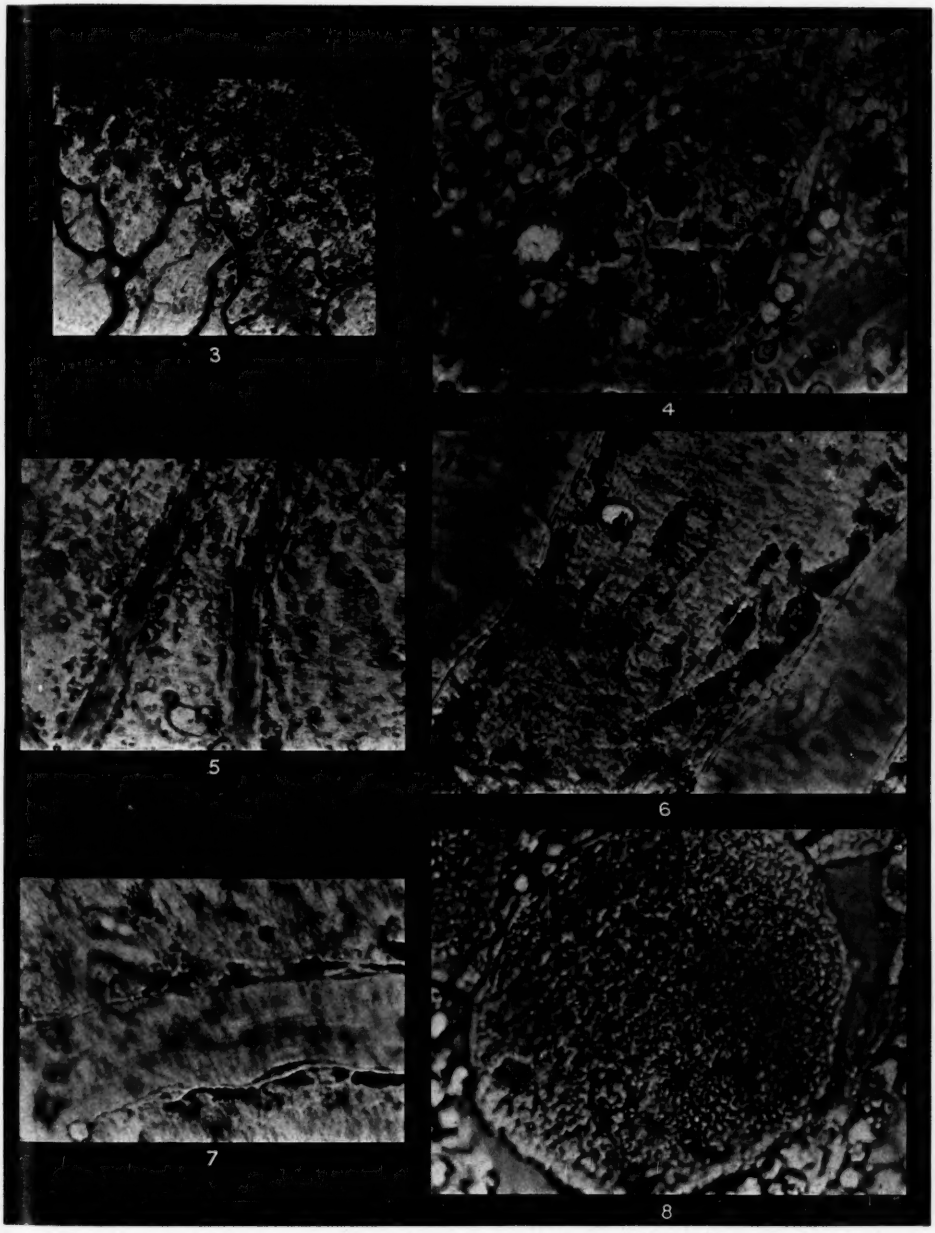
Figs. 46–51.

Reactions of a histiocyte in an injured region (× 510).

Fig. 46.—April 2nd. The cell is a typical histiocyte and it contains a small droplet of vaseline. The cell is on the central plug near the edge, and is in an injured region. New vessels have started to grow on to the plug. The tissue near the centre of the plug is dead.

Fig. 47.—April 4th. The histiocyte has changed its shape, but has not changed position.

Fig. 48.—April 5th. Capillary sprouts have grown into this region, but the vaseline-containing cell is still in almost exactly the same position it occupied April 2nd.



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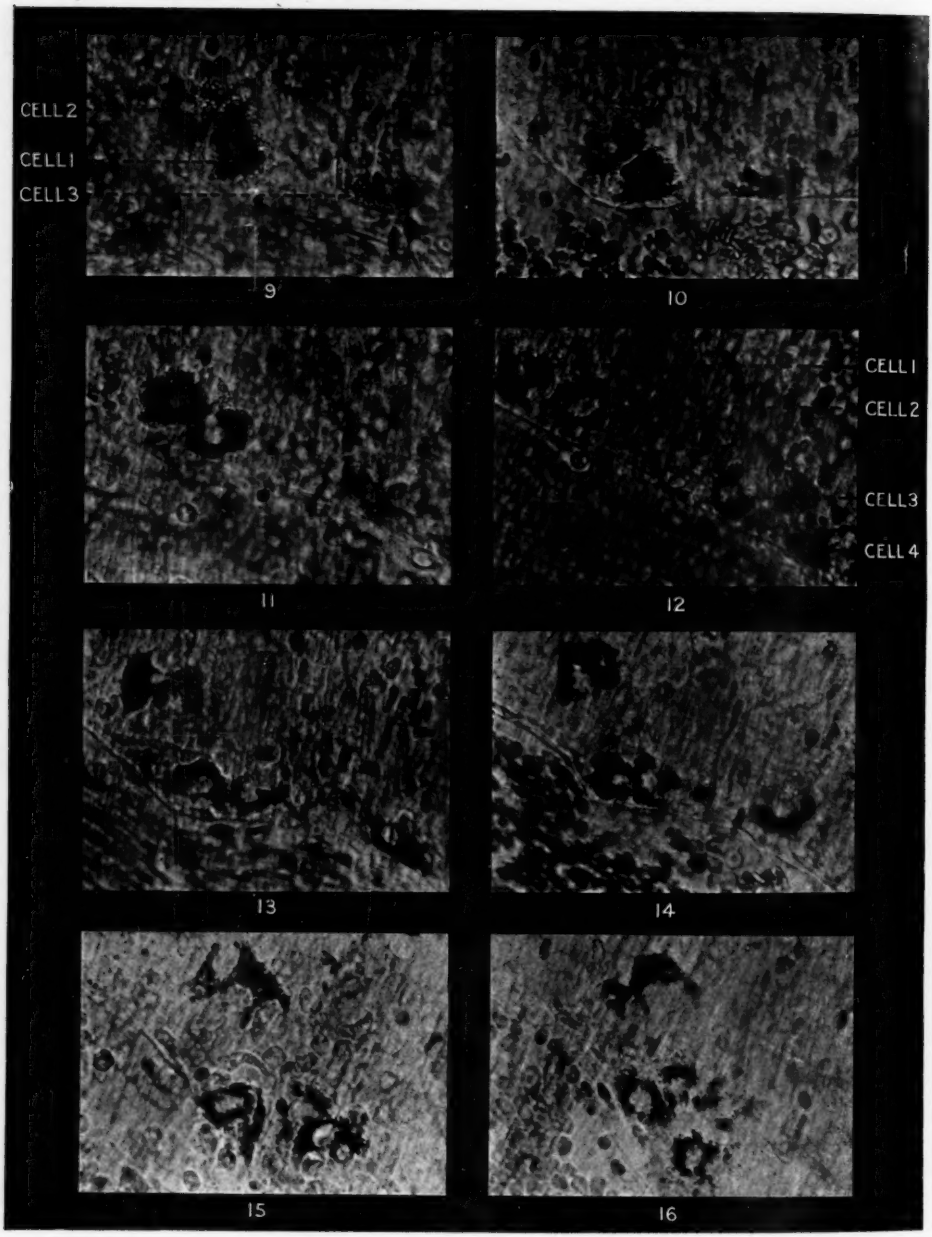

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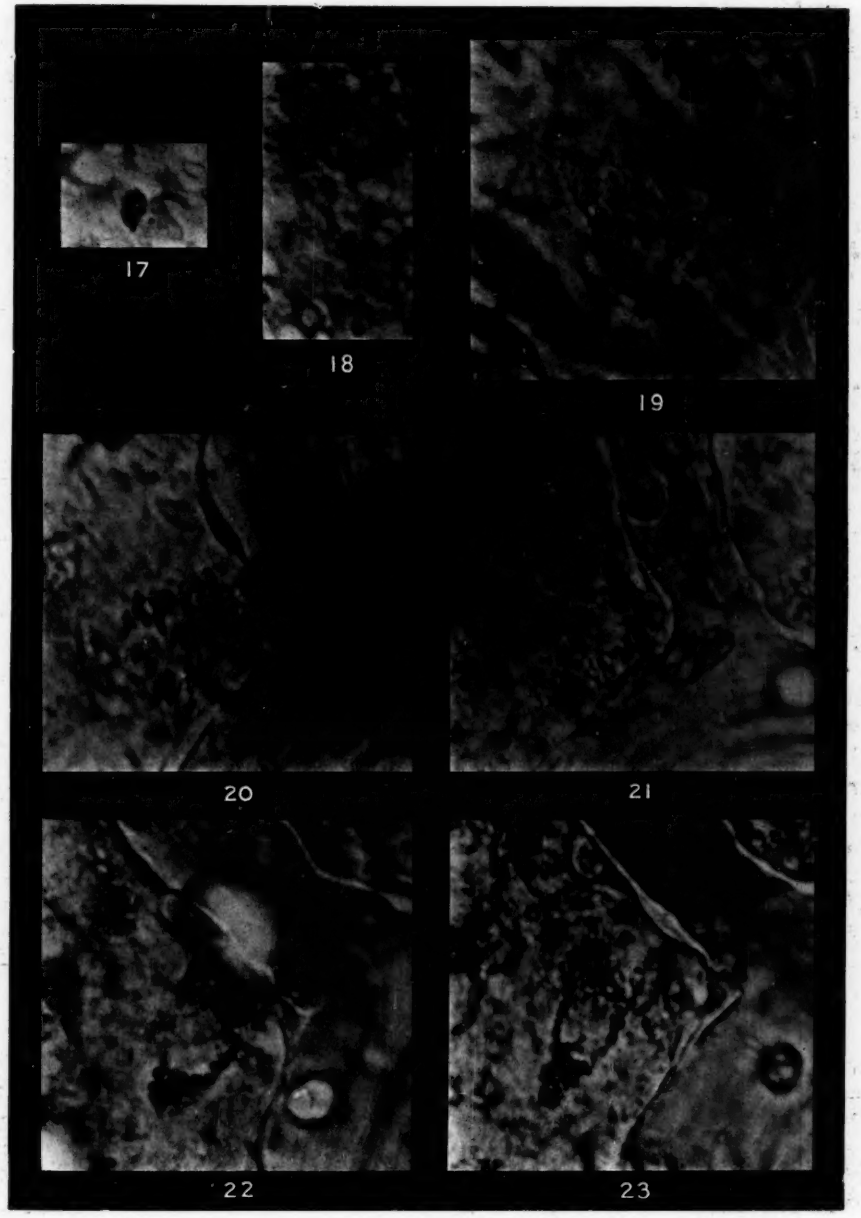

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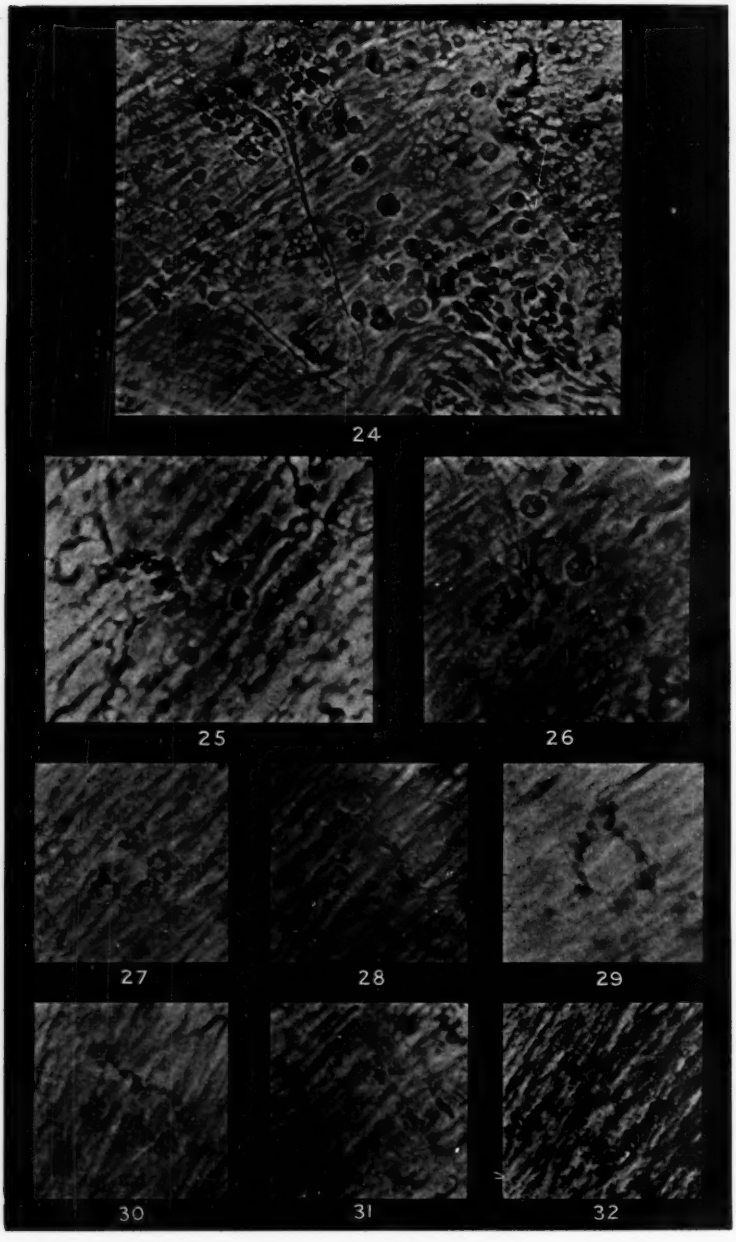

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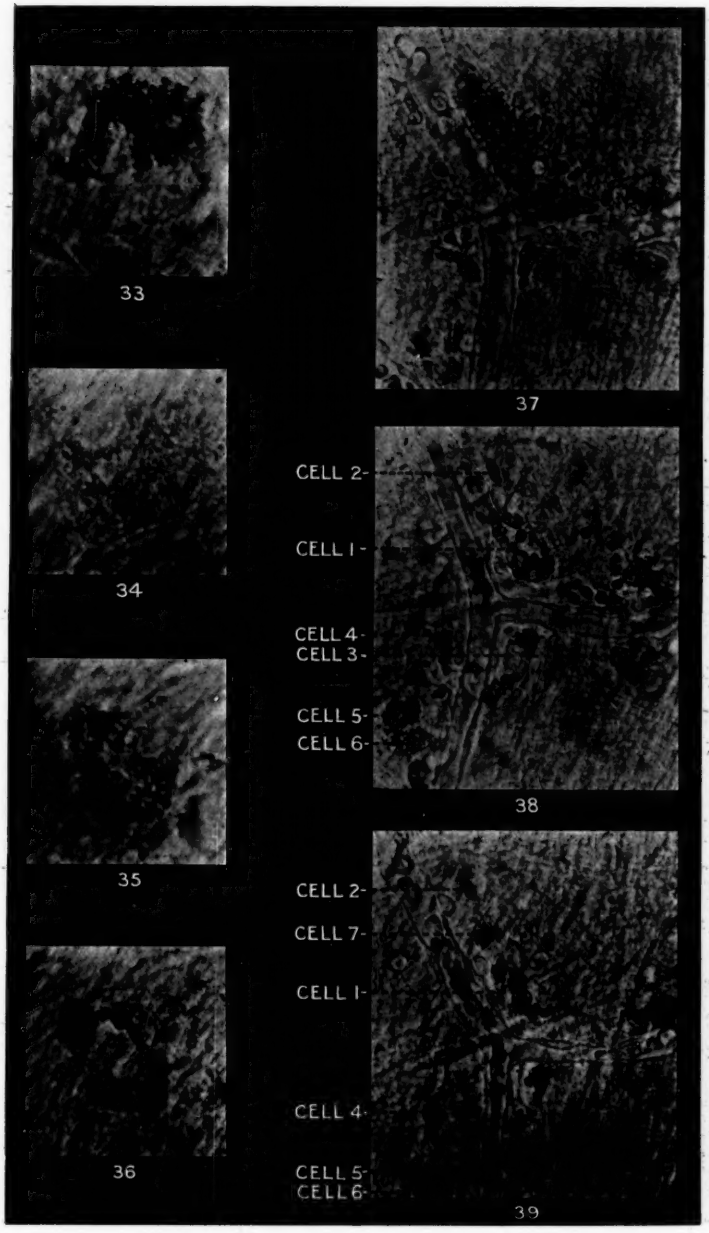

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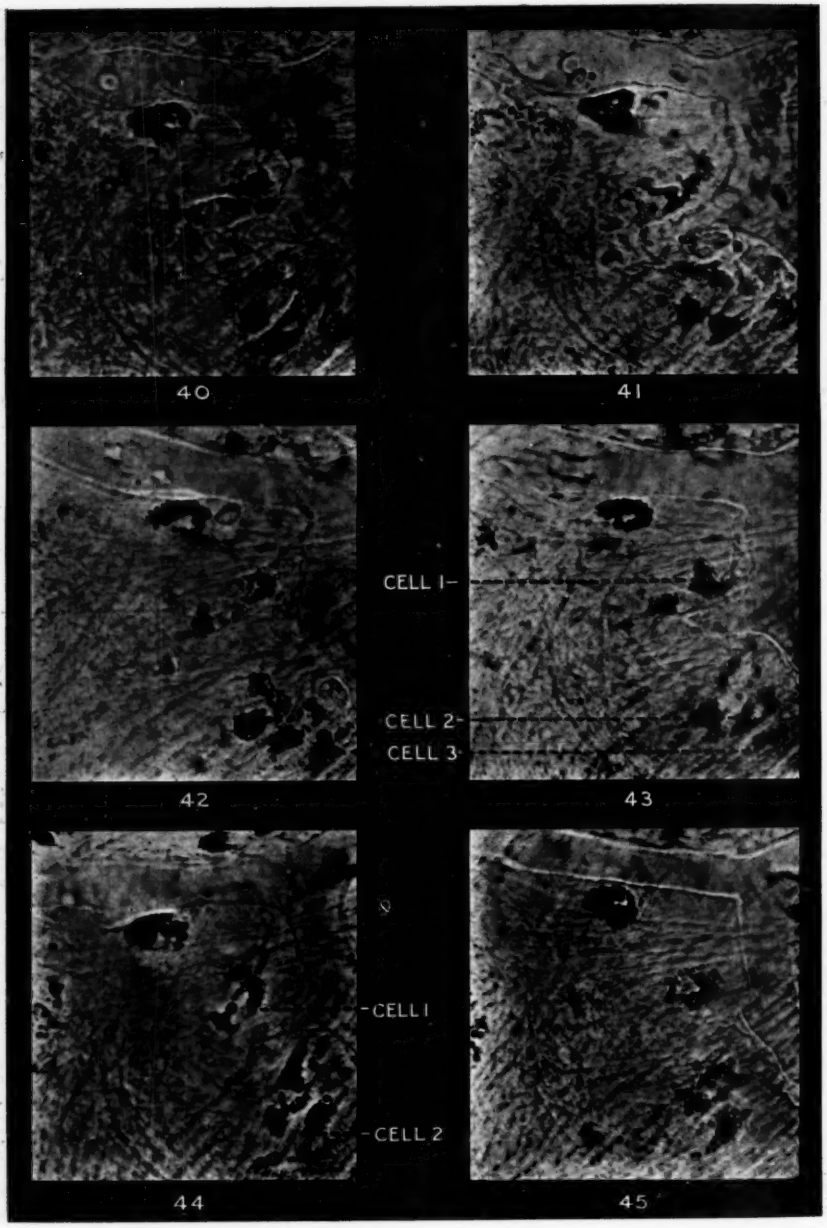

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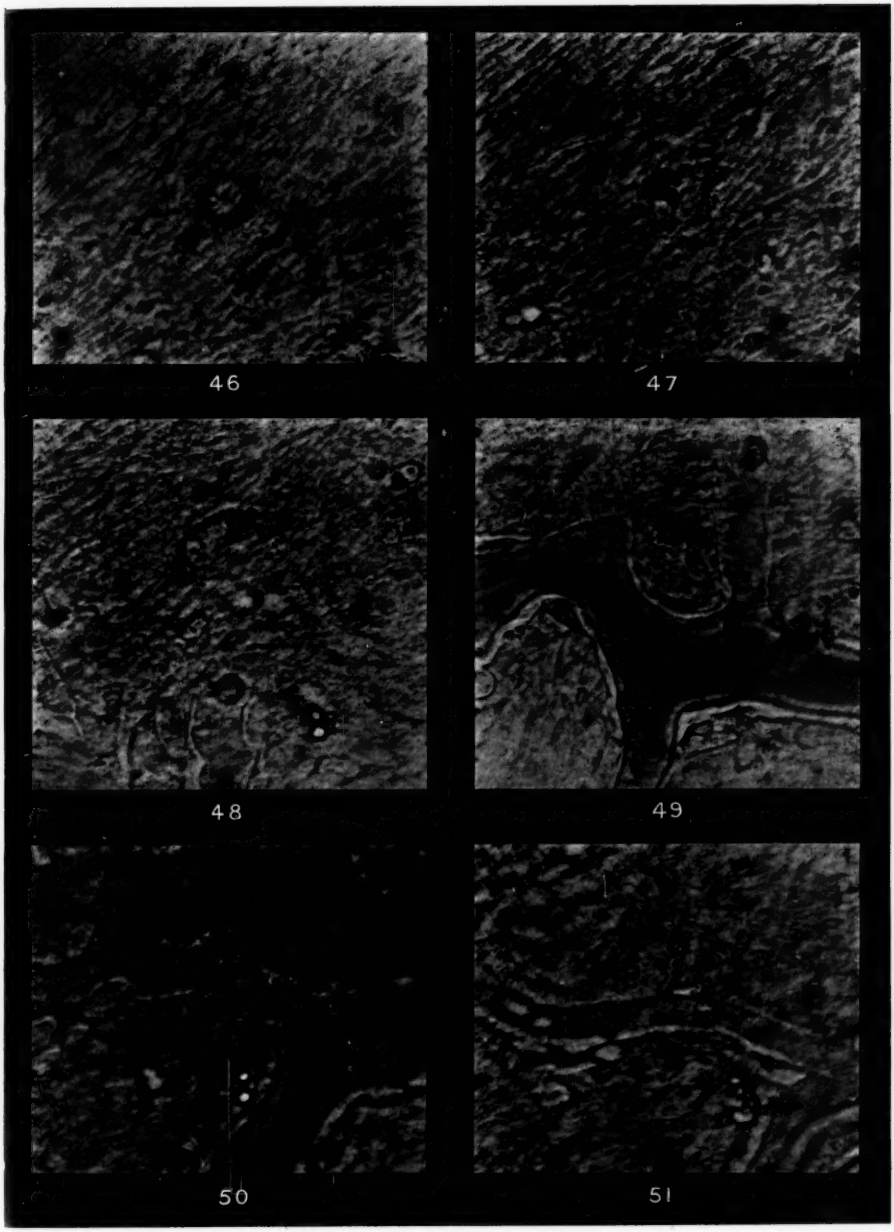

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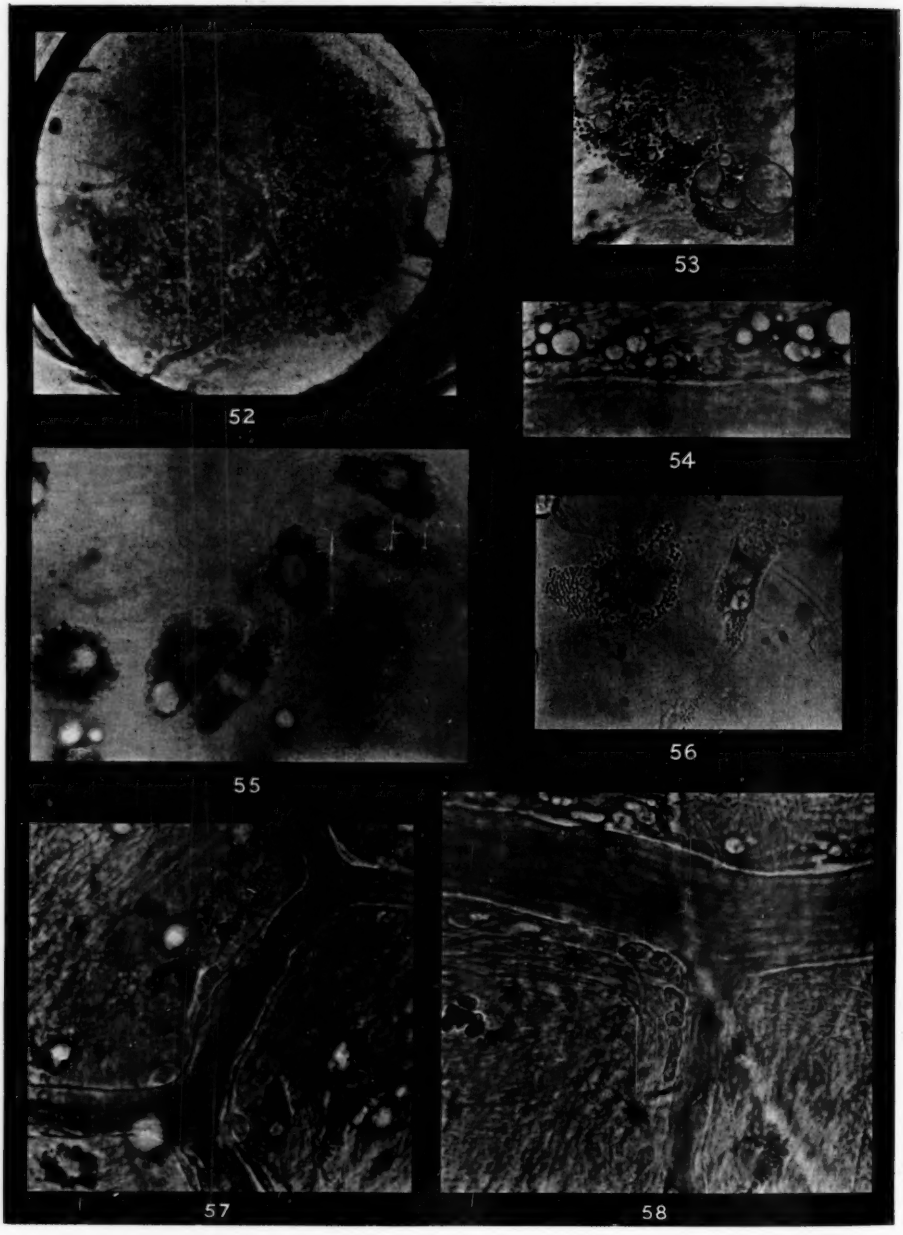

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Ebert and Florey.

Fig. 49.—April 6th. The vessels have actually grown past the vaseline-containing cell. The other cell seen in the photograph was present before in a slightly lower plane. It has moved out of the way of an advancing vessel.

Fig. 50.—April 7th. The vascular pattern has changed since yesterday and the vessels have grown well beyond the marked cell. The histiocyte has moved to a place above a vessel, but it is in the same relative position.

Fig. 51.—April 8th. The marked cell has divided and the daughter-cells are both typical histiocytes. There has been further growth of vessels, but they are in a slightly lower plane and are out of focus.

Subsequently these cells faded almost completely, but they never formed macrophages.

Figs. 52–58.

Fig. 52.—Macrophages laden with vaseline on the central pin (× 35). This photograph was taken seven months after the vaseline was pushed into the chamber. Four months later (May 22nd) the appearance of the central plug region was unchanged.

Fig. 53.—Two large cells containing droplets of vaseline (× 450). They are above a vessel.

Fig. 54.—Histiocytes containing numerous droplets of vaseline (× 450). These cells also contain granules of vital new red.

Fig. 55.—Macrophages on cover-slip which are changing into histiocytes (× 450).

Fig. 56.—Cell types frequently found in the semilunar spaces just at the edge of the table (× 450). The vital new red which has been taken up has a rather diffuse appearance and is concentrated at the centre of each cell, and there are many refractile granules at the periphery of each cell. These cells are similar in appearance to the epithelioid cells described by Lewis and Lewis (1926).

Fig. 57.—An adventitial lying between an endothelial nucleus and a well-stained histiocyte × 450).

Fig. 58.—Polymorphs in the perivascular spaces along vessels (× 450). Leucocytes which have emigrated into the tissues and have become relatively inactive often temporarily lie in the perivascular spaces along the large vessels.

DISCUSSION.

The techniques used in the past for the study of inflammation *in vivo* have required the comparison of transition forms between various cell types, and since this method is open to individual interpretation, there has naturally been considerable disagreement as to the origin of the mononuclear exudate cell or macrophage. Certain investigators have looked upon the monocyte as the stem-cell of the tissue macrophage, but other investigators have suggested almost every other type of cell of the blood and tissues as the precursor of the macrophage.

Many of the early students of the problem (von Recklinghausen, 1863; Ziegler, 1890; Grawitz, 1890; Marchand, 1890) believed that the exudate cells originated from local fixed tissue-cells, although Metchnikoff (1905), who made the first detailed study of the large mononuclears of the blood and fixed tissue phagocytes, grouped the two types together as functionally identical, and called them macrophages.

Another school believed the exudate cells had a double origin (Maximow, 1902, 1927; Ranvier, 1890, 1899–1900; Renaut, 1906–1907; Dominici, 1902). This group of investigators believed that lymphocytes and monocytes hypertrophied in the tissues to form macrophages, and at the same time, the "resting wandering cell" of Maximow, clasmatocyte of Ranvier, "cellules rhagiocrine" of Renaut or "cellules lymphoconjunctive" of Dominici were mobilized as the result of irritation and formed active macrophages, which became indistinguishable from the cells of hæmatogenous origin.

Still other investigators looked upon endothelium as the precursor of the

macrophage. Marchand's (1898–99) concept that the adventitial cell gave rise to the macrophage was extended by his student, Herzog (1916, 1923*a*, 1923*b*), who claimed that the adventitial cell originated from blood vascular endothelium. Mallory (1898), in a histological study of typhoid fever, concluded that the large mononuclear phagocytes characteristic of the disease originated from reticular cells in Peyer's patches, endothelium in blood-forming organs as well as from blood vascular and lymphatic endothelium. Later he came to believe that the mononuclear exudate cells always arise from endothelium.

Influenced by Mallory's conclusions other investigators (McJunkin, 1919 ; Foot, 1919–20, 1921 ; Permar, 1921) used carbon suspensoids such as indian ink in their studies of inflammation. They concluded that the ingestion of carbon particles is a specific reaction for cells of endothelial origin, and that blood vascular endothelium gave rise to macrophages locally. Lang (1926) repeated the experiments of Foot and McJunkin, and concluded that endothelium plays no part in the production of macrophages. Other investigators have consistently denied the activity of local endothelium in the production of exudate cells (Maximow, 1927 ; Lewis, 1925 ; Seeman, 1930 ; Clark and Clark, 1935). In our investigations we have found no evidence for the participation of endothelium in the production of macrophages.

Up until now the most convincing proof of the identity of the monocyte and the macrophage has come from tissue-culture experiments and *in vivo* observations on amphibians (Clark and Clark, 1930). Awrorow and Timofejewskij (1914) first demonstrated that macrophages and giant-cells could be derived from cells of the blood in tissue culture. They cultured blood from a leukæmic patient, and concluded that the transformation forms were derived from myeloblasts and lymphocytes. Later, Lewis and Lewis (1924–25, 1925, 1926) and Carrel and Ebeling (1922, 1926) showed that monocytes of normal blood changed into macrophages in tissue culture.

In hanging-drop culture of blood from elasmobranchs, teleosts, amphibia, reptiles, birds and mammals (including man), Lewis and Lewis (1924–25, 1925, 1926) found that in the course of a few days the large mononuclears of the blood changed into cells indistinguishable from tissue histiocytes, epithelioid cells and giant-cells. They concluded that all three cells are merely variations of the same cell type.

Carrel and Ebeling (1922) cultured buffy coat from hen's blood, and found that after a week they obtained a pure strain of mononuclears. This was repeated by Fischer (1925). Later Carrel and Ebeling (1926) compared cultures of chicken buffy coat with cultures of chicken subcutaneous connective tissue, and found that monocytes and macrophages cultivated in media of identical composition became identical in structure. They concluded that these two types of cells are functional variations of a single-cell type.

The observations on buffy coat cultures have been repeated by many investigators (Caffier, 1927 ; Vasiliu and Stoica, 1929 ; Katzenstein, 1931 ; Reeves, 1934 ; Jacoby, 1938). Reeves (1934) actually watched an individual monocyte hypertrophy and lose its typical rosette arrangement of neutral red granules. Jacoby (1938) compared the rate of growth of hen monocytes and hen-tissue macrophages *in vitro* and found that they behave very much alike under similar conditions.

Convincing evidence in favour of the identity of monocytes and macrophages in amphibians was presented by Clark and Clark (1930). Direct observations were made on the tails of living amphibian larvæ, and the Clarks found that the large mononuclears of the blood migrated into the tissues and changed into cells indistinguishable from large pigmented macrophages. They actually followed the change of individual blood-cells, marked with carmine, into pigmented macrophages.

Other experiments, although less convincing, provide additional proof for the potentialities of the monocyte. Eliot (1926) injected carmine-containing blood-cells from one rabbit into another. At the end of five days the injected rabbit was killed and carmine was found in epithelioid cells in the lung, in splenocytes and macrophages in the spleen, and in Kuppfer cells in the liver. Eliot concluded that the carmine-laden mononuclears of the blood had changed into these different cell types. Higgins and Palmer (1929) produced experimental hæmatomas in rabbits, and described the formation of macrophages within the hæmatomas. They concluded that the macrophages originated from monocytes and lymphocytes.

Cunningham *et al.* (1925) have consistently maintained that the monocyte of the blood and the tissue histiocyte are different cell strains. They believe the monocyte is derived from reticular cells and the histiocytes from endothelium. Their principal criterion for separating the two cell types is the different reaction to supravital dyes exhibited by each cell (1923–24, 1925; Sabin *et al.*, 1925). With neutral red the monocyte is said to stain with a characteristic rosette and mitochondria are said to be more numerous. In a critical review of the supravital technique, however, Hall (1938) concluded that it offers an inadequate criterion for differentiating between different cell types.

Erf (1937–38) studied supravital preparations of human blood, and found that degeneration vacuoles appeared in monocytes which gave these cells a " macrophagic " appearance. He concluded that the macrophages seen in tissue culture were degenerative forms and not true macrophages.

We have shown in this paper, however, that the changes observed in tissue culture by numerous investigators are not degenerative, but are changes which normally occur in inflamed tissue.

Ever since Aschoff and Kiyono (1913) showed that certain cells in the blood which they called histiocytes took up lithium carmine and indigo carmine, various investigators have suggested that macrophages are a normal or pathological constituent of blood.

Simpson (1922) described " macrophage showers " after repeated injections of certain colloidal dyes and suspensoids, and she found that the macrophages were unevenly distributed in the circulation, appearing chiefly in the right ventricle and blood from hæmatopoietic organs, particularly the liver and spleen. Numerous transition forms were present between monocytes and blood macrophages. Masugi (1927) failed to verify the shower phenomenon in vitally stained animals, although he found that monocytes in repeatedly stained animals were more like histiocytes when examined supravitally. Petersen (1925) described a large cell which he called an endothelial phagocyte, and which he believed was a normal constituent of human blood. Sabin and Doan (1926) studied supravital preparations of blood from rabbits and man,

and claimed they could distinguish a fourth cell type, the blood clasmatocyte, which they concluded was a normal constituent of rabbit and human blood.

It seems unnecessary to postulate another cell type to account for the macrophages which are occasionally found in the blood. Lewis (1925) made observations on the living lung of the frog and found, after injecting indian ink into the heart, that large ink-containing mononuclears lodged in the lung capillaries. These were ordinary blood monocytes, but after several hours they had hypertrophied and looked like macrophages. These cells did not come from the spleen or liver, because just as many appeared in the lung capillaries after the blood-supply from liver and spleen has been tied off. Sabin and Doan (1926) described the blood clasmatocyte as containing refractile granules which are not present in monocytes, but we have shown that refractile granules accumulate in monocytes after they migrate into the tissues.

Although most investigators, whatever they may consider the origin of the macrophage, assume that it ultimately becomes a resting histiocyte, none, so far as we know, have adequately described the changes in the living state which a macrophage undergoes in changing into a resting histiocyte.

Certain early investigators (Maximow, Renaut, Ranvier) believed that histiocytes were mobilized in inflammation. Maximow (1902) called the histiocyte the "resting wandering cell", and assumed that in inflammatory conditions it rounded up, became a "polyblast" and migrated into injured regions. This view has been accepted by many students of the problem (Bloom, 1938). Katzenstein (1931) repeated Maximow's (1902) experiments, and studied the exudate cells which migrated into celloidin chambers inserted in the stomach, walls of rabbits. In an animal vitally stained with carmine she found polyblasts containing carmine in the chamber and she concluded that these came from histiocytes. She gave no data, however, as to when or by what route the dye was given. If the dye was given during the course of the experiment it is obvious that it would be taken up by both monocytes and histiocytes, so that the presence of carmine in polyblasts would not necessarily mean that they were derived from histiocytes.

There is evidence that the pigmented macrophages found in amphibian connective tissue do migrate in response to the appropriate stimulus. Clark and Clark (1930) saw large pigmented macrophages migrate toward droplets of injected cream and ingest them, and Freund (1930–31) described the migration of large tissue-cells in the living tongue of the frog in response to a mechanical injury. Freund claimed that all the exudate cells in the frog's tongue originated from tissue-cells, but his evidence was not conclusive. He failed to give the times of observation, but apparently he watched only the very earliest stages of inflammation, and he says that high-power observations were difficult to make, so that much of the work had to be done on fixed and stained material.

Our observations show that at least under certain inflammatory conditions the histiocyte in the adult mammal does not migrate into an injured region, although it retains its phagocytic properties. There is certain supporting evidence for this in the literature. Cappell (1930) showed that if an animal was intensely stained with a vital dye, so that the histiocytes were loaded with dye-granules, and free dye was present in blood and tissue fluid but little

was present in the peritoneal cavity, the macrophages which appeared in the peritoneal cavity as the result of irritation showed very little staining. Sabin *et al.* (1925) injected cat's blood into the subcutaneous tissue of the rabbit and found that the normal histiocytes near the clot were not stimulated. Seemann (1930) claimed that the local histiocytes were mobilized in inflamed connective tissue and formed macrophages, but he found very few macrophages containing carmine in an animal locally stained with lithium carmine. We believe that much of the confusion as to the part actually played by local histiocytes in inflammatory conditions is due to the lack of facilities existing in the past for following individual cells throughout the inflammatory process.

We consider that the observations recorded in this paper furnish conclusive evidence for the views advanced by some of the authors quoted that the monocyte develops in the tissues through stages to a cell indistinguishable from a tissue histiocyte.

SUMMARY.

The extravascular development of the blood monocyte has been traced in a modified Sandison-Clark chamber in rabbits' ears.

The monocyte develops into a cell indistinguishable from a tissue histiocyte.

Vital dyes have been used to facilitate the observations.

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THE METABOLISM OF LACTIC ACID IN DIPHTHERITIC TOXÆMIA.

C. R. DAWSON* AND E. HOLMES.

From the Biochemical Laboratory, Cambridge.

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THE methods of biochemistry have long been applied to the study of the changes brought about by toxic conditions in the body, but the rapid development of our knowledge of metabolic processes has of recent years very much widened the field for investigation of this kind. In an earlier paper, one of us, with M. C. A. Cross (Cross and Holmes, 1937), published the results of an investigation of the effects of diphtheritic toxæmia upon the carbohydrate metabolism of the livers of rabbits, dealing in particular with the question of gluconeogenesis. Many of the experiments were performed on isolated liver slices, and the present work has been done in order to confirm and amplify, by study of the whole animal, some of the conclusions drawn from the experiments on isolated tissues; for in the present state of our knowledge such confirmation is highly desirable.

It was concluded, from the earlier experiments, that although in early toxæmia (*i. e.* 24 hours after the injection of a dose of toxin fatal in 48 to 60 hours) an injection of lactate would no longer give rise to deposition of glycogen in the liver, the power of the liver to form carbohydrate from lactate and other substrates (*i. e.* the power of gluconeogenesis) was but little impaired. The effect of toxæmia, arising from diverse causes, in preventing glycogen deposition is an old and often-repeated observation, and the literature is fully discussed elsewhere (Holmes, 1939). That failure to deposit glycogen may co-exist with an almost unimpaired power of gluconeogenesis was, however, a new finding. If it were correct, it should follow that, in early toxæmia, the injection of carbohydrate-forming substances such as lactate should give rise to a much greater hyperglycæmia than in the normal animal, for the sugar formed from the lactate, etc., instead of being stored in the liver, would be expected to escape into the blood-stream.

To test whether or not this was the case, the blood-sugar and blood lactic acid both of normal rabbits and rabbits which had received injection of diphtheria toxin were determined at intervals after the slow intravenous injection of *d*-lactate in doses of 1 g. per kg. of body-weight. The liver glycogen was determined at the end of a number of the experiments. In addition to measuring the blood lactate, blood sugar and liver glycogen, we determined

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the rate at which slices of liver both from normal and from 24-hour "toxic" rabbits caused lactate to disappear. We also investigated the effect upon blood sugar, blood lactic acid and liver glycogen of "eucortone" (adrenal cortical extract, Allen & Hanbury) after 24 hours' toxæmia.

METHODS OF INVESTIGATION.

In experiments in which diphtheria toxin was used, it was injected subcutaneously in amounts which were found to be fatal in from 48 to 60 hours. The required dose, for the specimen of toxin at our disposal, was obtained by diluting the toxin 480 times and by injecting 1·55 c.c. of this diluted toxin per kg. rabbit. Both normal and toxin animals were kept for 24 hours before the commencement of the experiment in a metal box with a coarse wire mesh lid, which was large enough to ensure their comfort but did not permit them to run about. Food was withheld, but water was given.

Blood sugar was estimated by the method of Hagedorn and Jensen (1923). In a number of experiments fermentable and non-fermentable reducing substance was measured. The values for the latter were 8–14 mg. per 100 c.c., which are similar to those found by other workers. They do not seem to be modified by injection of lactate or by toxæmia.

Lactic acid was measured according to the method of Friedmann, Cotonio and Shaffer (1927) after precipitation of the blood or liver slices by trichloroacetic acid. Glycogen was determined by the method of Good, Cramer and Somogyi (1933). The animals were anæsthetized by the intravenous injection of 1 c.c. per kg. of "numal" (Roche), the abdomen quickly opened and three samples of liver of about 1 g. weight rapidly cut with scissors, each from a separate lobe, and put into 2 c.c. of 30 per cent. potash in tared centrifuge tubes, which were then reweighed. Slightly higher values for liver glycogen are obtained when these precautions are taken than when the animal is killed without anæsthesia.

Blood samples were drawn from an ear vein, clotting being prevented with oxalate. When possible 2 c.c. of blood was obtained for each sample. Blood-sugar determinations were made in triplicate. Lactic acid was always determined in duplicate on the trichloroacetic acid filtrate. Some of the toxæmic rabbits yielded blood only with great difficulty, and on one or two occasions the final blood samples had to be taken with a needle and syringe from the inferior vena cava after the animal had been anæsthetized, and the abdomen opened to obtain the pieces of liver for glycogen determinations.

d-lactic acid was used for injection. It was neutralized with soda, phenol red being used as an indicator. The dose was 1 g. per kg. body-weight, and the final volume of the solution was adjusted so that it contained 100 mg. lactic acid (124 mg. sodium *d*-lactate) per c.c. Injections were made through a hypodermic needle into a marginal ear vein, by means of a large syringe actuated by a moving plunger. The plunger was driven by an electric motor, which was geared so that 10 c.c. of lactate solution containing 1 g. of neutralized *d*-lactate acid was delivered in 20 minutes. The rabbit sat quietly in the metal box throughout the experiment. Urine, if passed, escaped through a hole in the bottom of the box, which led to a metal cylinder, through which

it was delivered into a bottle. Any urine found in the bladder when the animal was killed was added to the sample. The quantity of acid or alkali necessary to bring 10 c.c. of the urine to pH 7·45 was determined, and its lactic acid content was estimated. Injection of lactate into a normal animal results in the excretion of additional alkali in the urine. It was thought that, if the metabolism of lactate were interfered with during toxæmia, less alkali would be excreted than is the case in a normal animal. This, in general, proved to be so. We found that 5–20 per cent. of the lactate administered was excreted in the urine. Toxæmia had no regular effect on this elimination.

In the earlier experiments, blood samples were taken at 20-minute intervals. But it was soon seen that the withdrawal at this frequency of the relatively large (2 c.c.) samples required caused a marked hyperglycæmia even in a normal rabbit which was receiving no injection. This hyperglycæmia was accompanied by a marked decrease in clotting-time of the blood. Samples were therefore taken at hourly intervals, and when this was done, the blood sugar of a normal rabbit which received no injection, or into which Ringer's solution was injected instead of lactate solution, remained perfectly steady throughout the experiment.

In work such as this, variability in the reaction of the animals to a dose of toxin always gives rise to difficulty: some of them are either more or less resistant than usual to the dose of toxin chosen, and as a result time and material are inevitably wasted. In an attempt to assess better the clinical condition of the animals during the experiments, continuous records were made of their body temperature by means of a thermocouple and galvanometer. The couple (copper-constantan) was placed in a hypodermic needle, which was inserted under the skin of the rabbit's back. The cold junction was kept in a thermos flask of water at about room temperature, and the deflections of the small mirror galvanometer were recorded on bromide paper rotating in a home-made camera. The apparatus was frequently standardized by inserting the needle containing the hot junction in water of known temperature. The rabbit's back was covered by a flannel " jacket ", as without this it was found that the skin temperature of the normal rabbit was subject to fluctuations. Less help was gained from the temperature records than was hoped, for the temperature rises soon after the injection of toxin, and remains somewhat elevated but remarkably constant until near death. One or two points were observed, however, which will be discussed in due course.

RESULTS.

Liver Glycogen of Normal Rabbits.

(a) *After lactate injection.*

Although it is well known that the injection of lactate gives rise to the deposition of liver glycogen, we thought it worth while to perform an experiment to discover the point at which maximum synthesis might be expected under our experimental conditions. Five rabbits were therefore starved for 24 hours. One was killed without receiving any injection, and the other four were killed 1, 2, 3 and 4 hours respectively after the commencement of an

injection of neutralized *d*-lactate. As in all other experiments, the lactate was administered at the rate of 1 g. in 20 min., and the total injection of lactate amounted to 1 g. per kg. body-weight. The results (Fig. 1) show that the maximum synthesis may be expected between the 2nd and 3rd hour. The glycogen is expressed here, as in other figures, as g. per kg. body-weight at death, since the ratio liver weight/body weight alters during the experiment.

(b) *After starvation.*

We also performed some experiments to determine the glycogen content of the liver after varying periods of starvation, and without any injection.

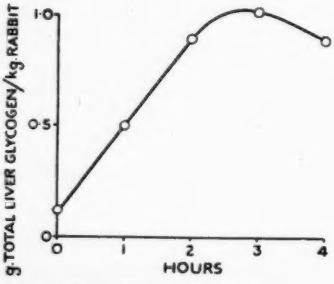

FIG. 1.—Deposition of liver glycogen after lactate injection into normal rabbits.

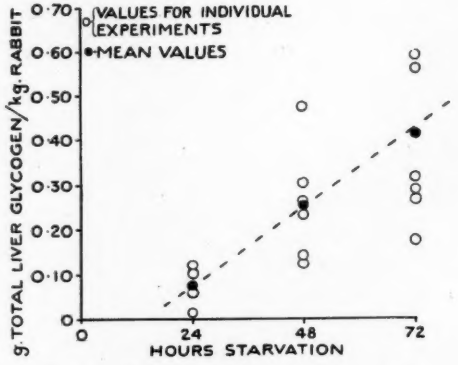

FIG. 2.—Change in rabbit liver glycogen on starvation.

We found a minimum value after 24 hours, after which there was a definite rise, at least until the third day. The results of a number of experiments are plotted in Fig. 2. The animals were fed on the ordinary laboratory mixed diet (bran and oats, cabbage, water). The figures are expressed as g. of liver glycogen per kg. body-weight. This result is in accordance with the findings of Mirski, Rosenbaum, Stein and Wertheimer (1938) on rats. These authors, in a most interesting paper, discuss the question of the relationship of carbohydrate reserves to fasting and to previous diet.

Comparison of the Effect of Injection of Lactate on the Blood Sugar, Blood Lactate and Liver Glycogen of Toxæmic and Normal Rabbits.

Since animals do not react uniformly to the same dose of toxin, it is necessary to treat our results statistically to make sure that they are significant. In doing so, however, it becomes unnecessary to present in full so large a number of individual experiments as would be the case if we relied merely upon the average figures obtained, and justified our conclusions by showing the details of the experiments from which the average was deduced.

TABLE I.—*Effect of Lactate Injection on Blood Sugar of "Toxic" and Normal Rabbits.*

Condition	Normal.	"Toxic."
Number of expts.	Starved 24 hr.	23–24 hr. toxæmia
Average wt. rabbits (g.)	7	10
Weight range (g.)	2100	2030
	1250–2700	1300–2575

Mg. blood sugar/100 ml.

	Normal.		"Toxic."		Comparison of normal and "toxic."		
	Mean (M.).	Probable error of mean (P.E.M.).*	Mean (M.).	Probable error of mean (P.E.M.).*	Mean difference (M.D.).	Probable error of mean difference (P.E.M.D.).*	Ratio: M.D./P.E.M.D.
Initial	102·5	±2·7	108·1	±2·5	+5·6	±3·7	1·51
Increase after:							
(hr.) 1	+16·8	±2·6	+19·9	±2·8	+3·1	±3·8	0·82
,, 2	+15·2	±3·2	+34·7	±3·6	+19·5	±4·8	4·06
,, 3	+8·6	±2·0	+43·8	±4·6	+35·2	±5·0	7·04

* The values for P.E.M. in the table are derived from the so-called standard deviation in the following manner:

$$\text{Standard deviation (S.D.)} = \sqrt{\frac{\Sigma\, d^2}{n-1}}$$

where (n) is the number of values used in computing the mean, and (d) is the difference between each value and the mean value.

$$\text{P.E.M.} = \frac{\text{(S.D.)}\ (0{\cdot}674)}{\sqrt{n}}$$

The probable error of the difference between two means M_1 and M_2 is given by

$$\text{P.E.M.D.} = \sqrt{(\text{P.E.M.}_2)^2 + (\text{P.E.M.}_1)^2}$$

The difference between two mean values (M.D.) is considered to be significant when that difference divided by the probable error of that difference gives a value in excess of 3.

TABLE II.—*Effect of Lactate Injection on Blood Lactic Acid of "Toxic" and Normal Rabbits.*

Condition	Normal.	"Toxic."
Number of expts.	Starved 24 hr.	23–24 hr. toxæmia
Average wt. rabbits (g.)	10	11
Weight range (g.)	2175	2076
	1525–2700	1300–2575

Mg. lactic acid/100 ml.

	Normal.		"Toxic."		Comparison of normal and "toxic".		
	Mean (M.).	Probable error of mean (P.E.M.).*	Mean (M.).	Probable error of mean (P.E.M.).*	Mean difference (M.D.).	Probable error of mean difference (P.E.M.D.).*	Ratio: M.D./P.E.M.D.
Initial	18·7	±0·72	34·9	±4·6	+16·2	±4·7	3·45
Increase after:							
(hrs.) 1	+48·8	±4·1	+111·4	±8·1	+62·6	±9·1	6·88
,, 2	+6·9	±1·6	+59·7	±8·1	+52·8	±8·3	6·36
,, 3	+4·5	±1·5	+83·1	±8·6	+79·6	±8·7	9·15

* See footnote to Table I.

Tables I and II and Figs. 3 and 4 show the results obtained when the blood sugar and blood lactic acid are determined immediately before, and at hourly intervals for three hours after, the intravenous injection of neutralized *d*-lactic acid as previously described. Table III gives the value for liver glycogen obtained at the end of the three-hour period. It was regularly observed that the livers of animals which had received toxin were enlarged when compared with those of animals which had been starved for 24 hours, but which were otherwise normal. For this reason, the figures for glycogen, if expressed merely as a percentage, would not yield a true comparison; since the animals also varied in size, we have expressed the values as g. liver glycogen per kg. body-weight. Since most of the animals used for the toxic values had previously been used (3 or 4 days earlier) as normals, we have comparatively

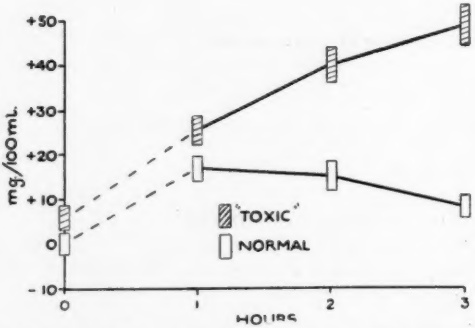

FIG. 3.—Change in blood sugar after lactate injection. (In Figs. 3, 4, 6 and 7 the curves are drawn through the mean values. The vertical heights of the rectangles represent the magnitude of the probable error of the mean in both directions.)

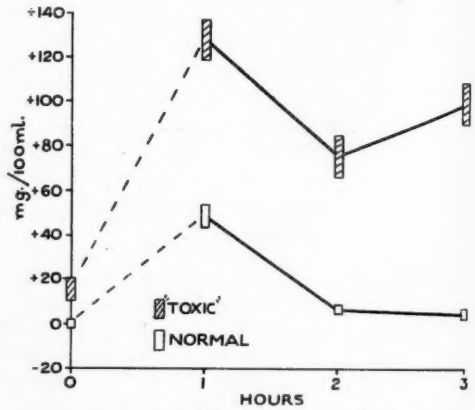

FIG. 4.—Change in blood lactate acid after lactate injection.

few " normal " values for glycogen. But we are concerned only with the fact that the " toxic " values are much lower than the " normal " ones, and it did not seem worth while to sacrifice more animals merely to demonstrate the already well-known fact that lactate injection into a normal animal is followed by the deposition of glycogen in the liver.

The values both for blood sugar and blood lactic acid for the first, second and third hours after the injection are shown as increases above the initial value. Both in the " normal " and the " toxic " animals the blood sugar is raised at the end of the first hour, and the difference between the increase in the two cases is not statistically significant, since the ratio Mean/Probable error of Mean is less than 3·0. By the same criterion, the small increase in the initial blood-sugar value shown by the " toxic " animals over the " normals " has no significance. In the second and third hours, however, the increases in the case of the " toxic " animals are marked, and are undoubtedly significant.

Moreover (Fig. 3) the blood sugar of the toxic animals is steadily rising, while that of the normals is falling to the initial level and has almost reached that value after 3 hours. The results of the blood lactic acid determinations (Table II and Fig. 4) are still more striking. All values, including the initial ones, are significantly raised in the "toxic" as compared with the "normal" animals.

From these results it is clear that the injection of lactate causes a prolonged rise of blood sugar in the "toxic" animal which is only temporary in the "normal". This is in accord with the hypothesis that in early toxæmia, although lactic acid can be converted to carbohydrate, the latter cannot be stored as glycogen and glucose is shed into the blood-stream. This does not indicate that a failure to store glycogen is the sole cause of the hyperglycæmia. A failure on the part of other tissues to remove carbohydrate either by storage or by oxidation might play a part. It is well known that glucose tolerance and the response to insulin is disturbed in toxæmia (for literature see Holmes, 1939), and though disturbance of hepatic function may play a predominant part in this, it is not certain that it is alone responsible, since there might also be interference with carbohydrate oxidation. If, however, the latter were true, it might be expected that the fasting blood sugar would be definitely increased, for a slower removal of carbohydrate by the tissues without any accumulation in the liver (see Table III) would obviously result in the accumulation of glucose in the blood. But hyperglycæmia, though sometimes recorded, is not a prominent or constant feature of early diphtheritic toxæmia, while in late toxæmia hypoglycæmia is the rule. On the other hand, the results of our lactic acid determinations do (Fig. 4) make it clear that failure to deposit glycogen is not the only effect of the toxæmia, for the rate of removal of lactic acid from the blood-stream is markedly slowed. The blood lactic acid is definitely higher in the third than in the second hour. This phenomenon is very constant, and we cannot at present suggest any other explanation than that it is perhaps due to the fact that the toxæmia is a progressive process, and that the animals' condition was therefore steadily deteriorating during the three hours of the experiment.

TABLE III.—*Glycogen Values after Injection of Lactate into Normal and "Toxic" Rabbits. Results, expressed as g. total liver glycogen/kg. body-wt., 3 Hours after the injection of* 1 *g. neutralized* d*-lactic acid/kg. body-wt.*

	Normal.	"Toxic."
Condition	Starved 24 hr.	23–24 hr. toxæmia
Number of expts.	3	9
Average wt. rabbits (g.)	1760	1988
Weight range (g.)	1210–2200	1300–2575
Mean ratio: liver-wt./body-wt.	0·028	0·037

	Normal.		"Toxic."	
	Mean (M.).	Probable error of mean (P.E.M.).	Mean. (M.).	Probable error of mean (P.E.M.)
Glycogen: g./kg. body-wt.	0·618	..	0·065	±0·016

By no means all the lactic acid which is metabolized by liver slices *in vitro* is converted into carbohydrate : some of it is oxidized (Cross and Holmes, 1937), and a further proportion is converted into some substance or substances of an acidic nature not yet identified. Cross and Holmes found that, although 24 hours' toxæmia had little effect on the carbohydrate synthesis, it did reduce the oxygen consumption of liver slices, and also the rate of removal of acid radicles as determined by manometric methods.

Effect of Lactate Injection on Body Temperature.

It has been already explained that, although continuous records were made of the temperatures of a number both of normal and of toxic animals, the results proved less useful than was hoped. One interesting point, however, emerged. The injection of lactate into a normal animal is invariably accompanied by a sharp rise in temperature, and this rise was absent or much reduced

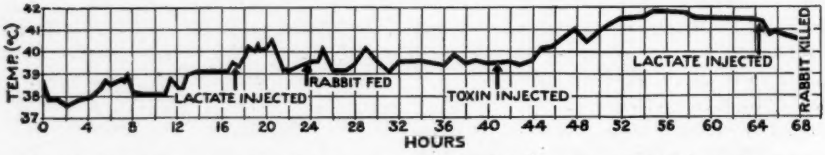

FIG. 5.—Temperature record. Diphtheria toxin and lactate injected at the points indicated.

in the case of animals previously injected with toxin, except in a few cases in which it was clear, from the other data, that the animal was more resistant than usual to the action of toxin. Injection of lactate is followed by an increase in oxygen consumption of the whole animal (Buchwald, Cori and Fisher, 1933). The mere observation of a rise in temperature is not, of course, absolute proof of an increase in the rate of metabolism, for the temperature depends upon the balance between heat production and heat loss, and it would be necessary to measure the total heat production before it could be confidently stated that the presence or absence of such a rise indicated an alteration in the rate of metabolism ; the phenomena might be caused simply by an alteration in the mechanisms for heat loss. Nevertheless, our observations on this point seem worth mention. Fig. 5 shows the plot of a typical temperature record. Before the injection of toxin the lactate caused a sharp rise in temperature, which was entirely absent when lactate was again administered 24 hours after the toxin had been given.

While the original hypothesis, to test which this work was undertaken, has been satisfactorily verified, certain animals in our series, the data for which have not been included in Tables I or II, behaved in an unexpected way, being obviously either less or more resistant to the toxin than the remainder. We found their behaviour very instructive. In one instance, a rabbit which had received the usual dose of toxin died in convulsions, probably hypoglycæmic in nature, shortly after the third hour from the beginning of the

injection of lactate. The blood sugar fell steadily instead of rising after the lactate injection, while the blood lactate rose.

Two other animals (24 " toxæmic ") showed a rising blood sugar with a steady or falling blood lactate and appreciable deposition of liver glycogen at the end of three hours. They behaved in a similar way to an animal which was used only 15 hours after toxin injection, and were no doubt more resistant to the action of toxin than most animals. One of them further behaved abnormally in that it eliminated as much as 30 per cent. of the injected lactate in the urine ; the amount usually eliminated was from 5 to 20 per cent.

In Vitro *Removal of Lactate by Liver Slices.*

Since there was a very marked delay in the rate of disappearance of injected lactate from the blood, it was obvious that although Cross and Holmes (1937) had not observed any marked failure on the part of the liver to convert lactate to carbohydrate, there was, nevertheless, a very definite disturbance of the power to deal with lactate. This disturbance need not necessarily be in the liver, since lactate is oxidized by other tissues of the normal animal. On investigating, however, the power of isolated liver slices to cause the disappearance of lactic acid *in vitro*, we found an unmistakable alteration in this respect in the liver from " toxic " as compared with " normal " animals. In performing the experiments, the animals were stunned and bled, and the livers rapidly removed and weighed. Slices were cut with a razor-blade, and accumulated in a moist chamber. After samples of the required size had been weighed, the slices were suspended in 3 c.c. Ringer's solution buffered with 0·025 *M* $NaHCO_3$ (Krebs and Henseleit 1932), and containing 0·2 per cent. neutralized *d*-lactate. The tissues were shaken at 38° C. in an atmosphere of 95 per cent. O_2 and 5 per cent. CO_2. The " initial " samples were removed from the bath after equilibration for 10 min., and treated with trichloro-acetic acid ; the " final " samples were shaken for a further period of 1 hour. The amount of tissue used was 200 mg. with normal livers, and 300–400 mg. with " toxic " livers. Duplicate experiments were always put up and duplicate estimations performed on each sample. The results (Table IV) were remarkably uniform. Since the livers from " toxic " animals were heavier than those from normal ones, the results are expressed as g. lactic acid removed by the liver per kg. body-weight per hour. They leave no doubt that the removal of lactate by the liver is seriously affected in 24-hour toxæmia, whether other tissues are involved or not. Cross and Holmes (1937) have published similar results on the removal of lactate by liver tissue from 48-hour " toxic " and " normal " animals. The difference was even more marked than in the case of the present less severely " toxic " animals. The rate of removal found for normal livers was, however, slower than that observed in the present experiments. We attribute this to the fact that Cross and Holmes used racemic lactic acid for their experiments, whereas for the present work *d*-lactic acid has been employed, and also that these workers used larger amounts of tissue and continued their experiments for two hours. They probably, therefore, did not measure the maximum rate of lactate removal.

TABLE IV.—*Removal of Lactic Acid by Slices of Liver from Normal and "Toxic" Animals. (Results expressed in g. lactic acid removed by the liver per kg. of body-wt. per hr. All animals starved for 24 hr.)*

	Normal.	"Toxic" (23–24 hours).
Number of experiments*	5	8
Average wt. of animals (g.)	727	901
Mean ratio: liver-wt./body-wt.	0·030	0·039
Mean lactic acid removed (g./kg./hr.)	0·233	0·145
Probable error of mean (P.E.M.)	±0·006	±0·010

Comparison of normal with "toxic":	
Mean difference (M.D.)	0·088
Probable error mean difference (P.E.M.D.)	±0·012
Ratio: M.D./P.E.M.D.	7·34

* Each experiment was performed in duplicate.

Effect of "Eucortone".

Previous work by many different authors has suggested that in diphtheritic, and perhaps also in other forms of toxæmia (for literature see Holmes, 1939), an important, and perhaps the most important, cause of the disturbance of carbohydrate metabolism is a lesion in one or more of the endocrine glands. The results have been most commonly attributed to a lesion of the suprarenals, and from time to time claims have been advanced that patients and experimental animals can be successfully treated by the administration of suprarenal cortical extract. We have therefore carried out a number of experiments in which we injected suprarenal cortical extract ("eucortone") into "toxic" rabbits, and observed the effect upon the blood sugar, blood lactic acid and liver glycogen after the injection of lactate. Earlier experiments with M.C.A. Cross (Cross, 1938) upon the effect of "eucortone" upon liver glycogen had given equivocal results; on account, however, of the high cost of the material only moderate sized doses had been employed. In the present experiments, massive doses were used comparable to those used by Britton and Silvette (1934) in demonstrating the effect of the hormone on normal rats; these doses amounted to 17 c.c. of extract (representing 75 g. of gland per c.c.) per kg. body-weight. They were injected intraperitoneally 2 hours before the beginning of the lactate injection. We would express our thanks to Messrs. Allen & Hanbury for making it possible for us to obtain the necessary amount of extract.

With these doses there was a distinct effect (Figs. 6 and 7; Tables V, VI and VII); the initial blood-sugar level was considerably higher than the usual initial level either of "toxic" or of normal animals; on the other hand, it had risen comparatively little by the end of the first hour after the injection of lactate, and thereafter remained steady. The blood lactic acid had risen to about the same level as in untreated "toxic" animals by the end of the first hour, but thereafter fell towards (though not as far as) the level found in normal animals. The liver glycogen was significantly higher than in the untreated "toxic" animals, though still much below that found in normal animals. It

will be seen that no observations are given for the second hour ; in order to economize " eucortone " we used the smallest available rabbits, and therefore omitted to draw the 2-hour blood sample in order to avoid disturbing the experiment by withdrawing too much blood.

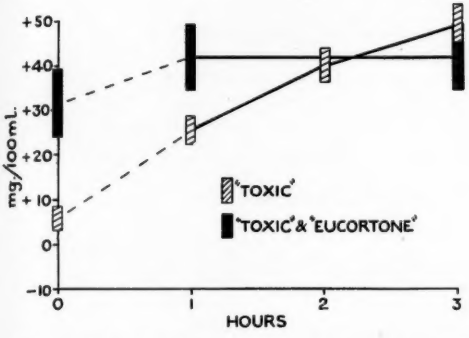

FIG. 6.—Change in blood sugar after lactate injection in "toxic" animals and " toxic " animals treated with " eucortone ".

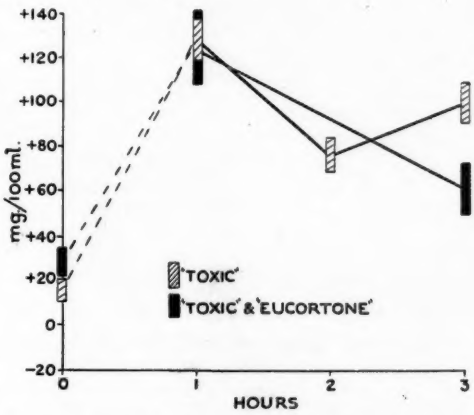

FIG. 7.—Change in blood-lactate acid after lactate injection in " toxic " animals and " toxic " animals treated with " eucortone ".

TABLE V.—*Effect of Lactate Injection on Blood Sugar of " Toxic " Rabbits with and without Treatment with " Eucortone ". All animals* 23–24 *hr. " toxic ".*

	Toxic.	" Toxic " injected with " eucortone ".
Number of expts.	10	10
Average wt. rabbits (g.) . . .	2030	815
Weight range (g.)	1300–2575	703–1200

Mg. blood sugar 100 ml.

	" Toxic."		" Toxic " injected with " eucortone ".		Comparison of " toxics " with " toxics " injected with eucortone.		
	Mean (M.).	Probable error of mean (P.E.M.).	Mean (M.).	Probable error of mean (P.E.M.).	Mean. difference (M.D.).	Probable error of mean difference (P.E.M.D.).	Ratio: M.D./ P.E.M.D.
Initial . .	108·1	2·5	133·9	±7·8	+25·8	±8·2	3·15
Increase after :							
(hr.) 1 .	+19·9	±2·8	+10·7	±6·8	−9·2	±7·4	1·24
,, 2 .	+34·7	±3·6	..	..	..	..	..
,, 3 .	+43·8	±4·6	+10·7	±7·7	−33·1	±9·0	3·67

The values 3·15 and 3·67 in the last column indicate that the initial blood-sugar values and also the 3-hour increase in blood-sugar level are significantly different for the two sets of animals. However, at the end of the first hour the difference in increase of blood-sugar level is not statistically significant.

TABLE VI.—*Effect of Lactate Injection on Blood Lactic Acid of "Toxic" Rabbits with and without Treatment with "Eucortone". All animals 23–24 hr. "toxic".*

	"Toxic."	"Toxic" injected with "eucortone".
Number of expts.	11	10
Average wt. rabbits (g.)	2075	815
Weight range (g.)	1300–2575	703–1200

Mg. lactic acid/100 ml.

	"Toxic."		"Toxic" injected with "eucortone".		Comparison of "toxics" with "toxics" injected with "eucortone".		
	Mean (M.).	Probable error of mean (P.E.M.)	Mean (M.).	Probable error of mean (P.E.M.).	Mean difference (M.D.).	Probable error of mean difference P.E.M.D.	Ratio: M.D./ (P.E.M.D.).
Initial	34·9	±4·6	46·3	±5·8	+11·4	±7·4	1·54
Increase after:							
(hr.) 1	+111·4	±8·1	+95·6	±15·8	−15·8	±17·8	0·89
,, 2	+59·9	±8·1	..	..	..	..	..
,, 3	+83·1	±8·6	+33·4	±11·2	−49·7	±14·2	3·50

The mean blood lactic acid values for the two sets of animals are significantly different only at the 3-hour period.

TABLE VII.—*Glycogen Values after Injection of Lactate into "Toxic" Rabbits with and without Treatment with "Eucortone". Results, expressed as g. total liver glycogen/kg. body-wt., 3 hours after injection of 1 g. neutralized d-lactic acid/kg. body-wt. All rabbits 23–24 hr. "toxic".*

	"Toxic."	"Toxic" treated with "eucortone".
Number of expts.	9	10
Average wt. rabbits (g.)	1988	814
Weight range (g.)	1300–2575	703–1200
Mean ratio: liver wt./body-wt.	0·037	0·041

	"Toxic."		"Toxic" treated with "eucortone".	
	Mean (M.).	Probable error of mean (P.E.M.).	Mean (M.).	Probable error of mean (P.E.M.).
Glycogen: g./kg. body-wt.	0·065	±0·016	0·224	±0·044

Comparison of "toxic" with "toxic" treated with "eucortone":

Mean difference (M.D.)	0·159
Probable error of mean difference (P.E.M.D.)	±0·047
Ratio: M.D./P.E.M.D.	3·38

Since we desired to determine the liver glycogen, our experiments yield no information as to whether the treatment with "eucortone" would have prolonged the animals' lives. No obvious change in their condition was apparent. The literature leaves one with the impression that though endocrine disturbances probably play an important part, particularly in early toxæmia, they are by no means the only factors at work. This view has been fully

discussed elsewhere (Holmes, 1939). Toxæmia, as well as disturbing endocrine function, certainly causes changes in the cells of the liver and other tissues which alter their capacity to carry out their normal metabolic functions; these changes are fully as important as alterations in endocrine secretions. The present findings are in accordance with such a view, though a fuller investigation of the effects of suprarenal cortical hormone would be desirable. If given earlier, in divided doses, or in even larger doses than we were able to use, it might prove more effective. To such an investigation, however, the present high cost of the material imposes a serious bar. A necessarily limited number of experiments have shown that "eucortone" does not increase the rate of removal of lactic acid added to isolated liver slices *in vitro*, either when the animal received an injection of the hormone two hours before it is killed, or when the hormone was added to the fluid in which the tissue was suspended. The specimen of "eucortone" used for these last experiments was specially put up without preservative.

DISCUSSION.

Previous workers (Hartmann and Senn, 1932*a*, *b*, *c*; Soffer, Dantes, Newburger and Sobotka, 1937*a*, *b*; Soffer, Dantes and Sobotka, 1938) have investigated the effects of the injection of lactate into normal human subjects, and into patients suffering from hepatic disease and other conditions. They have observed that in cases of hepatic disease there is a delay in the disappearance of lactate from the blood-stream.

Minot and her collaborators (Minot, 1931; Minot, Dodd and Saunders, 1934) have shown that there is an accumulation of lactic acid in the blood and urine of animals poisoned with carbon tetrachloride and with guanidine, and that both these conditions are accompanied by necrosis of the liver. Lactic acid is known to accumulate after poisoning with phosphorus and chloroform, and Yannett and Darrow (1933) have recorded an increase in blood lactic acid in diphtheritic toxæmia in rabbits. Our results, therefore, agree with those of other workers in indicating a severe disturbance in ability to deal with lactic acid when the liver is damaged.

SUMMARY.

1. The slow intravenous injection of neutralized *d*-lactic acid into starved, but otherwise normal, rabbits results in a temporary rise in blood sugar and blood lactic acid values. After 3 hours both blood sugar and blood lactic acid values are again close to normal. During this period glycogen is deposited in the liver, the maximum deposition taking place between the second and third hour.

2. Similar injections into rabbits in early diphtheritic toxæmia (23–24 hours' duration) results in a significantly increased and prolonged rise in the blood-sugar level, even though the disappearance of lactic acid from the blood is considerably slowed. After 3 hours practically no glycogen is found in the liver: the rise in blood sugar is attributed to failure in the power to deposit glycogen without a corresponding failure in the power of glyconeogenesis.

3. The power of isolated liver slices to cause the disappearance of lactic acid *in vitro* is markedly decreased in early toxæmia.

4. The injection of large doses of "eucortone" into rabbits in early toxæmia 2 hours before the lactate injection causes a significant, although far from complete, amelioration of the usual effects of the toxin upon glycogen deposition and upon the rate of lactic acid disappearance from the blood.

5. It has not been possible to demonstrate any effect of "eucortone" on the rate of disappearance of lactate when shaken *in vitro* with slices of liver from "toxic" animals.

It is a pleasure to acknowledge the help which we have received from Mr. H. Mowl, both in the performance of the experiments and in making a large number of histological preparations.

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Authors' Errors.

A. T. Fuller and W. R. Maxted, p. 178, line 17, "mg. per c.c." should read "mg. per 100 c.c.".
C. G. Pope, p. 137, Table II, protein undigested (per cent.), "32" should read "72".

SURVEY OF PAPERS.

POPE and LINGGOOD have investigated the ratio of theoretical toxin nitrogen to coagulable nitrogen found in a number of crude diphtheria toxins and toxoids and have shown the presence of a large amount of coagulable protein, other than toxin protein, in such products. By using a special medium of low nitrogen content they have been able to reduce the amount of this contaminating protein which can be almost completely eliminated from the resulting toxoids by treatment with charcoal. From such charcoal filtrates it has been found possible to secure toxoids of a purity of 1500 L*f* units/mg. nitrogen by simple methods involving no acid precipitation (p. 297).

ANDREWES and SMITH have attempted to analyse the factors which determine the efficacy of influenza virus vaccines. The detrimental effect of tissue extracts from foreign animal species, especially when inactivated vaccines are employed, makes one fear difficulties in satisfactorily immunizing man. It is suggested, however, from analogy with work on ferrets, that the practical difficulties may be less when it is only attempted to reinforce a basic immunity such as most human adults possess against influenza (p. 305).

MONTGOMERY studied the immediate effect of small intravenous doses of mercuric chloride in rabbits and found that although there was no microscopic evidence of renal damage in the two hours of the experimental period, the injections produced circulatory collapse and proteinuria with a fall in the blood serum protein occurred within 15 minutes. The proteinuria quantitatively always exceeded the loss of serum protein and the results suggest that the latter is replaced continuously from the tissue depôts. Intravenous saline had no effect upon the shock syndrome (p. 316).

From plants infected with potato virus "Y", an insect-transmitted virus, BAWDEN and PIRIE have isolated a liquid crystalline protein with many properties similar to those of potato virus "X", although it is much less stable. The isolation is complicated by the small amount of virus in infective sap and by the presence of other constituents with properties in common with the virus (p. 322).

MCILWAIN has shown that pantothenic acid is necessary for growth of *Streptococcus hæmolyticus*. This substance is an amide of a dihydroxyvaleric acid and β-alanine, was first characterized as a nutrient for yeast by R. J. Williams in 1933, since when it has been implicated in the nutrition of certain bacteria, of the chick, and of the rat. This work is a further instance of the wide applicability of nutritional studies in a restricted field (p. 330).

FILDES and GLADSTONE confirm the finding of McIlwain *et al.* that glutamine is an essential growth factor for *Str. hæmolyticus*. It functions in vitamin-like concentrations. The general conclusions of the authors suggest that glutamine has a wider nutritional significance than as a factor for streptococci, and that its dispensability or otherwise as a nutrient depends upon the powers of synthesis of the bacteria tested (p. 334).

EBERT and FLOREY, using a modified Sandison Clark cell, have watched the extravascular development of individual monocytes into cells indistinguishable from tissue histiocytes. They produce evidence to show that macrophages appearing in organizing tissue do not originate from the histiocytes previously present in surrounding normal tissue (p. 342).

DAWSON and HOLMES using intact animals confirm earlier findings that in diphtheritic toxæmia there is a failure in the power of the liver to store glycogen with no corresponding failure in the formation of carbohydrate from non-carbohydrate sources. They show also a diminution in the power to remove lactate from the blood-stream when injected intravenously.

They demonstrate partial restoration of the power of the liver to form glycogen, following massive doses of suprarenal cortical hormone (p. 357).

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